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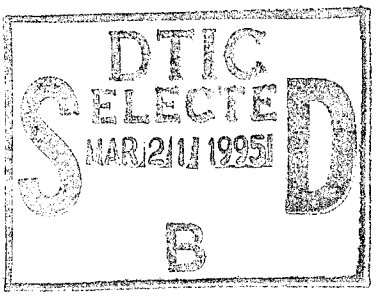

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Contract DAMD17-92-C-2504, Support of HIV interventional trials, was initiated in October of 1992 and is administered by Dr. Owen S. Weislow of SRA Technologies, Inc.. This contract is intended to provide both research and development support of clinical studies in HIV antiviral chemotherapy, immunotherapy and immunoprophylaxis. A number of significant contributions to WRAIR's mission have been made by this contract during FY94. The most important of these is the extension of the 215 ARMS assay for genotypic resistance to a number of patients in a large-scale, nationwide clinical trial to assess the significance of this approach. A comparison of this genotypic approach with the classical phenotypic assays of drug resistance as described in this report will doubtedly demonstrate the usefulness of this assay for the clinical management of patient therapy. SRA has also developed an in vitro assay for the evaluation of antiviral gene constructs in established cell lines and is actively pursuing a system that will enhance the efficiency of transduction in PBMCs that will permit the study of primary isolates. Such a system will prove invaluable for the longterm culture and ex-vivo treatment of patient cells with antiviral genes. Finally, the efforts of this contract have resulted in the development of an improved system for the rapid and cost effective phenotypic analysis of resistance to antiviral drugs as described in the previous section.

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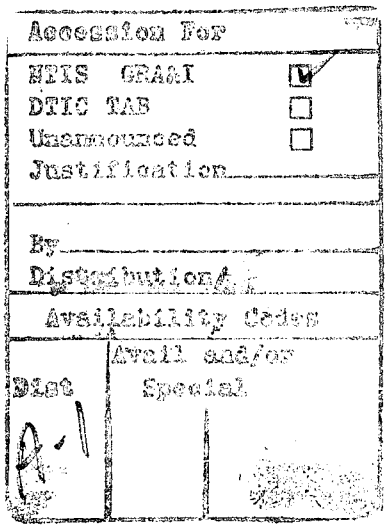

Aaron E. Wessler 10/29/94
PI - Signature Date

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INTRODUCTION

Contract DAMD17-92-C-2504, Support of HIV interventional trials, was initiated in October of 1992 and is administered by Dr. Owen S. Weislow of SRA Technologies, Inc.. This contract is intended to provide both research and development support of clinical studies in HIV antiviral chemotherapy, immunotherapy and immunoprophylaxis. The primary objectives of the contract, as stipulated in the contract award, are a) to develop assays to evaluate the viral and immune responses to anti-retroviral therapy including neutralization assays and drug susceptibility assays using clinical HIV isolates, b) to develop and validate assays that predict or demonstrate disease progression for use in interventional trials with an emphasis on molecular biologic approaches to viral characterization and quantification of viral burden, and c) to improve the accuracy, reliability, and cost effectiveness of clinical laboratory tests for all stages of HIV-1 and other retroviral infections. The contractor has, in consultation with the contract office's representative (Dr. Douglas L. Mayers, Capt, USN), incorporated three working groups to develop and optimize the necessary assays in support of these clinical studies and to establish production-level protocols. The three groups include the molecular biology working group, the cellular phenotype working group and the antiviral drug working group. The work scopes of each are briefly described below with a detailed discussion of the progress made by each. A Third section, the data group section, consisting of computer personnel from SRA, has been established to support the efforts of SRA to improve delivery of all necessary data elements to WRAIR personnel as stipulated in the contract award.

During FY94 the contractor expanded on the first year's development efforts and moved many assay systems into the production phase of operations. The impetus for this shift in focus stems, in part, from WRAIR's participation in new clinical and research protocols and a desire to apply new knowledge to patient management. Included in this arena are the molecular biology working group assays for the aa215 mutation of reverse transcriptase (RT) and sequencing of RT for drug resistance. In addition, we have provided a large-scale capability for the extraction of nucleic acids for use in viral burden studies conducted at WRAIR. The cell phenotyping group has scaled up studies of SI/NSI isolates along with antiviral gene construct evaluations and large scale expansions of viral stocks. The antiviral drug working group continues to evaluate clinical isolates for drug sensitivity as part of the RV43 drug resistance protocol and has, at the same time, evaluated many new compounds emanating from WRAIR and collaborating laboratories.

On the other hand, research and development continues to be a significant part of this contract's effort. Quantitative PCR is not quite ready for large-scale use and development is continuing in this area. The 215 mutational assay was modified during the last year and new assays, such as the 74 mutation for DDI resistance, are being developed by the molecular biology

working group to provide clinicians with an additional tool. The cellular phenotyping group participated in the development and evaluation of a new infectivity reduction assay employed in virus neutralization studies and has been studying the neutralization kinetics of laboratory and field isolates of HIV with an eye toward improving existing assays. This same group is attempting to improve on procedures currently employed for the transduction of antiviral genes in PBMCs and collaborating with WRAIR and the National Naval Medical Research Laboratories in evaluating long term cultures of purified CD4+ cells for *ex-vivo* use in investigations of genetic therapies. Finally, the antiviral drug group has helped develop a new rapid screening system for drug resistance that could reduce the time to phenotype patients undergoing antiviral drug treatment.

PROGRESS REPORT

1. Molecular Biology Working Group

During the second year (FY 1994) of this contract we concentrated on further improving the PCR assay used to test for the presence of AZT-resistance associated mutations at HIV-1 Reverse Transcriptase amino acid's 215 position. In addition, we began application of this technology to a new clinical protocol (244) sponsored by the AIDS Clinical Trials Group (ACTG), NIAID and WRAIR. We have also begun efforts to develop a similar mutational assay for the 74 mutation that confers DDI resistance. Finally, we have made improvements in our diagnostic DNA sequencing protocols for use in support of drug-resistance monitoring in clinical trials. Information on the rational behind these protocols as well as background information is provided below along with examples of the data generated using each of these methodologies and followed by copies of current protocols for each.

215 ARMS PCR Assay

Of great importance, in WRAIR clinical trials supported by SRA Technologies, is the monitoring of the acquisition of drug resistance. At this point in time, AZT (Zidovudine) is the primary drug used in the these trials. While potentially providing some benefit to the patient, the usefulness of AZT is limited by the tendency for almost all recipients to develop some level of resistance to the drug during the course of therapy. In order to better assess the effectiveness of treatment modalities, it is useful to have a rapid screening assay for patients that will indicate the onset of genotypic mutations associated with AZT resistance. During the FY94 contract year the 215 ARMS PCR assay was improved and applied to a nationwide clinical protocol for prosective analysis of the 215 mutation in clinical practice.

Amplification Refractory Mutation System (ARMS)

The theoretical basis for this assay was included in last years progress report. Briefly, a combination of ASO (Allele Specific Oligonucleotide) techniques and PCR has been developed that makes use of the best aspects of both techniques. Variously named the Amplification Mutation Refractory System (ARMS) or PCR Amplification of Specific Alleles (PASA), this technique takes advantage of the inability of synthetic oligonucleotide primers that are incompletely hybridized to a template to serve as effective PCR primers[1]. First described by Markham et al. and Sommer et al.[2,3], this technique has been applied to the detection of single base changes and identification of specific alleles associated with disease in such diverse instances as cystic fibrosis[4,5], phenylketonuria[3], apolipoprotein genotyping[6] and HLA typing[7]. Larder et al. have recently applied this technique to examine AZT resistance acquired during chemotherapy, first, by characterizing the genetic mutations in the HIV RT gene that can be linked to in vitro resistance[8], and more recently by applying this technique to the direct determination of the presence or absence of these mutations in patient blood samples[9]. Further work by his group has validated and extended this approach [10-13].

In addition to the drugs currently available, a number of new agents are being developed and tested as HIV chemotherapeutic agents against both the HIV RT gene[14,15] and other viral targets such as the integrase protein[16], and the HIV protease[17]. It is expected that as the new agents and combination therapies are administered to patients, new mutations conferring resistance to these agents will also be discovered. It will be useful to monitor the appearance of resistant virus in patient populations in order to adjust the therapeutic regimes in use at the time and that is the intent of the ACTG's 244 protocol. Although the details of the assay described below are for the detection of the mutation at amino acid 215 that confers AZT resistance, this procedure is readily adaptable to the detection and monitoring of mutations at other locations within the viral RT gene simply by changing the primers used in the second (nested) PCR reaction and re-optimizing the PCR reaction conditions (if needed) to maximize sensitivity. This has been done for the 74 mutation associated with DDI resistance and our efforts in this regard are also documented below.

Our most recent incarnation of the 215 ARMS protocol for the detection of AZT resistant genotypes is described below. Modifications from that which was described in last year's report include changes to the protocol's pelleting of virus, RNA extraction, RT and PCR master mixes, conditions of both rounds of PCR, dilutions of the first round PCR into the second round PCR, elimination of the common primer and the process of evaluation for sample mixtures. These changes were incorporated either after additional R&D supported the changes or when our experience with the ACTG clinical protocol indicated such modifications were desirable or required.

ARMS Protocol

PCR Reactions - 215 Mutation Detection

We use A(35) and NE1(35) primers for the first set of cycles and the B and either 215M or 215W primers for the second set of cycles to detect mutant (resistant) or wild type (sensitive), respectively. These primers are identical to those described by Larder and Boucher (B. Larder, personal communication[18]). A third primer, Control (C) was designed that lacked the terminal discriminating nucleotides found in the 215W or 215M primers. This primer was intended to serve as an amplification control for the second (nested) PCR step. However, we have now eliminated use of the common primer for the assay currently in production since no amplification problems with the second PCR have been incountered in more than 600 assays run since the contract's inception. The primer sequences in use today are given below. The NE1(35), 215M and 215W are 5' biotinylated.

A(35)	TTGGTTGCACTTTAAATTTTCCCATTAGTCCTATT
NE1(35)	CCTACTAACTTCTGTATGTCATTGACAGTCCAGCT
B	GGATGGAAAGGATCACC
215M	ATGTTTTTTGTCTGGTGTGAA
215W	ATGTTTTTTGTCTGGTGTGGT

The PCR cycle part of the assay is identical whether the source material is plasma or tissue culture supernatants (viral RNA), or patient PBMCs or co-culture cells (proviral DNA). However, serum samples and samples treated with heparin have proven to be somewhat difficult to handle and 40 cycles has been the standard for those samples. Sample preparation steps are given for each substrate. New protocols for all steps of the ARMS assay are provided.

Sample Preparation: PBMCs or Co-cultured Cells

1. Thaw frozen cells at 37°C and transfer to a sterile 15 ml polypropylene centrifuge tube.

2. Wash once with 10 ml PBS (2000 rpm 15 min.). Decant supernatant after wash and discard.

3. Add lysis buffer (10 mM Tris 8.3, 50mM KCl, 2.5mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20, proteinase K at 120 µg/ml) and resuspend pellet well for a cell concentration of 7.5 X 10^6 cells/ml. Be sure to lyse a negative control with the cell samples. Vortex briefly.

4. Incubate at 55°C - 60°C for 1 hr. Vortex before transfer in next step.

5. Transfer to 1.5 ml screw-cap microcentrifuge tube.

6. Heat-inactivate the proteinase K by incubating the tubes at 95°C for 15 min.

7. Transfer tubes to ice. Store lysates either at 4°C (no more than overnight) or at -20°C for longer periods.

Sample Preparation: Viral RNA from Plasma

Preparation of Plasma from Whole Blood

1. Centrifuge the whole blood at approximately 200 X g (1400 rpm) in a tabletop centrifuge at 4°C.

2. Remove the supernatant, taking care not to disturb the cell layer.

3. Centrifuge the supernatant from Step 2 at approximately 1000 X g (4000 rpm) in a tabletop centrifuge at 4°C. The purpose of this step is clarification, to remove residual cells.

4. If the plasma is to be extracted immediately, maintain on ice. If it is to be stored, aliquot into 1.0 ml cryo-vials, and store at -70°C.

Pelleting of Virus

1. If the sample is frozen, thaw quickly at 37oC, then maintain on ice.

2. Add 0.5 ml PBS/BSA to 1.5 ml screw cap tube. Tubes should be pre-labelled with specimen number.

3. Transfer 1 ml of plasma to each tube. Be sure to include negative control.

4. Pellet the virus by centrifugation at ≈12,000 x g (approximately maximum speed in a typical microcentrifuge at 4°C for 1h.

RNA Extraction

1. Remove and discard supernatant from the 1.5 ml tube by decanting and removing as much of the supernatatn as possible while the tube is inverted. Be careful not to disturb the pellet. Gentle tapping on clean gauze will help remove supernatant.

2. Add 800 µl Tri-Reagent (guanidinium/phenol). Vortex 15 s.

3. Allow to sit at least 5 min. at room temperature.

4. Add 160 µl $CHCl_3$ to each tube. Vortex 15 s.

5. Allow to sit at least 3 min. at room temperature.

6. Centrifuge at maximum speed (approximately 12,000 X g) in a microcentrifuge at 4°C for 15 min.

7. Remove the aqueous (upper, colorless) phase to a fresh tube.

8. Add 400 µl of cold isopropanol (IPA, 2-propanol) and 4 µl of 2.5 µg/µl tRNA to each tube. Mix well by vortexing.

9. Maintain at -20°C overnight.

10. Centrifuge at maximum speed in a microfuge at 4°C for 15 min.

11. Decant the supernatant.

12. Wash the pellet with 1 ml of ice-cold 75% ethanol. Vortex briefly.

13. Centrifuge at maximum speed (approximately 12,000 X g) in a microfuge at 4°C for 15 min.

14. Decant the supernatant.

15. Air dry the pellet. Do not use a Speed-Vac.

16. Add Virus Lysis Buffer A (1% NP-40, 0.04 mg/ml tRNA, 0.4 U/µl RNasin, 2 mM DTT) for 55 µl per 1 ml original specimen volume. Vortex.

17. Maintain at 42°C approximately 15 min. to fully dissolve the RNA, after which the extract should be stored at -70°C. The RNA should not be heated above 60°C until after reverse transcription, as RNasin in the Virus Lysis Buffer will be inactivated.

215 PCR Reaction Setup

A. DNA PCR:

The first set of PCR cycles uses A(35) & NE1(35) primers (NE1(35) primer is biotinylated) to produce a 805 bp fragment encompassing virtually all currently known drug resistance associated mutations in the HIV RT gene (amino acids 5-254 of RT).

PCR master mix:

7.1 µl H2O
22.4 µl dNTP (280 µM dNTP) (use dUTP only in the second PCR)
10.0 µl 10X 215 PCR buffer
10.0 µl Primers [A(35) & NE1(35); 250ng each.]

49.5 µl store at -20oC if required.
Add Taq polymerase (Promega, 5 units/µl) when ready to use.

When ready to begin PCR, aliquot 50µl of PCR mix with the Taq polymerase added to each reaction tube.

Add 70.0µl of oil overlay (may be omitted for PE 9600 cycler)
Add 50µl of sample lysate

NOTE: In contrast to the PCR protocol in use for HIV detection in our laboratory, dUTP and UNG are not used in the first PCR of this nested set. The use of dUTP significantly reduces the discriminating power of the 215W and 215M primers used in the second PCR reaction.

The 10X 215 PCR buffer contains:

500 mM Tris 8.3
250 mM KCl
15 mM $MgCl_2$
1 µg/ml BSA

1. In the positive control lab, add positive controls (10^4 sensitive and/or resistant cells) from freshly thawed stock dilutions to the appropriate PCR tubes.

2. Immediately carry the reactions to the cycler.

PCR Cycling Conditions for Perkin Elmer 9600 Cycler

First (Outer) PCR Reaction Cycling Conditions

1. 94oC 1' 15"
2. 94oC 30"
3. 55oC 30"
4. 72oC 2'
5. Repeat steps 2-4 for 18-30 cycles
6. Soak 72oC 10 min.
7. Soak 4oC

The precise number of cycles depends somewhat upon the expected number of infected cells. When testing co-cultured cells, 18 cycles is usually sufficient, due to the large number of infected cells in the population, whereas primary patient cells often require 30 cycles, while up to 40 cycles may be used to generate more product if needed for cloning and sequencing. Since there is no UNG in these reactions, they may be maintained at 25°C after last cycle until products are ready to be carried into second PCR. Yield of PCR products may be monitored by running a 10 µl aliquot of the first PCR reaction on an agarose gel. It should be noted that in cases of low numbers of virus, such as seen in patient PBMCs, no band may be visible after the first PCR. This is not necessarily an indication that the PCR failed however.

Reaction Setup for Second (Nested) PCR

The second (nested) PCR reaction utilizes the B and either 215W or 215M primers to discriminate between the wild type (AZT sensitive) or mutant (AZT resistant) genotype at amino acid 215 of the HIV-1 RT gene. The 215W primer recognizes either the Phe or Tyr mutant at amino acid 215 approximately equally.

1. Remove 10 µl of the **1st PCR reaction** and dilute 1:10 to 1:100 in water.

NOTE: The exact dilution can be varied to ensure clean discrimination between 215M and 215W primer products. In general, there is less than a 100X difference in the product yield between completely homologous primer/template combinations (sensitive virus DNA with 215W primer for example) and mis-matched primer/template combinations (sensitive virus DNA with 215M primer) (Frank White, unpublished observations). Because of this, if the quantity of product transferred into the second PCR (B & 215M/215W) is too high, cross-reactive bands appear in both sensitive and resistant reaction lanes. In this case, it is necessary to either dilute the products of the first PCR reaction further and repeat the second PCR, or repeat the first PCR with reduced cycle numbers. This latter approach generally gives slightly cleaner results, but is also more time and labor intensive. The second PCR is set up exactly as the first, with the addition of 10 µl of first reaction product and 90 µl of master mix containing the B and 215M or 215W primers. Note that dUTP can be substituted for dTTP in this reaction without affecting the specificity or sensitivity of the PCR. Uracil-N-glycosylase (UNG) may then be added to facilitate contamination control, as is the standard procedure for HIV detection PCR reactions.

PCR Master mix for second PCR (B & 215M/215W primers)

10.0 µl dNTPs (250 µM dNTP. May substitute dUTP for dTTP)
10.0 µl 10X 215 PCR buffer
10.0 µl primers (B & 215W, 215M) at the ratio of 1:2 total of B+215M or W should equal 250 ng/reaction
3.0 µl $MgCl_2$ (25 mM stock, 2.25 mM final concentration)

33.0 µl Store at -20°C until needed.

Add 56.3µl of H2O, 0.2 µl UNG (0.2 units, Epicentre Technologies) and 0.5 µl Taq polymerase (Promega, 5 units/µl) per reaction when ready to use.

When ready to begin PCR aliquot 90.0 µl total of the above mix to each reaction tube a 70.0 µl oil overlay (may be omitted for PE 9600 Cycler) and 10.0 µl diluted products from first PCR.

NOTE: There are slight changes in the concentrations of some of the reaction components (2.25 mM $MgCl_2$) between the first and second PCR reactions. These conditions have been optimized to increase product yield of the second PCR reaction.

Second (Nested) PCR Reaction Cycling Conditions

1. 25°C 3'
2. 94°C 5'
3. 94°C 1'
4. 48°C 30"
5. 72°C 30"
6. Repeat steps 2-4 for 30-40 cycles. Soak at 72°C after last cycle until products are either stored (-20°C) or analyzed.

B. For RNA PCR:

1. 215 RT PCR Master Mix:

DEPC H2O	3.2 µl
5X RT Buffer	10.2 µl
0.1 M DTT	2.0 µl
0.05 µg/µl NE1' primer	5.0 µl
10% NP-40	3.4 µl
25.0 mM dNTP's	1.0 µl
Total	24.6 µl/reaction

Store in -20°C freezer. Add 0.2 µl of RNasin (40 units/ml) and 0.2 µl of MMLV-RT per reaction when ready to use.

a. Add 25 µl of each RNA sample to a 96 well plate.

b. Overlay with 20 µl of mineral oil, seal and place in a heat block at 75°C for about 5 min. Immediately place on ice or at 4°C.

c. Add 25 µl of RT mix to each well.

d. Use either a 9600 cycler or manual transfer between heat blocks set for the following temperatures:

42°C 30 min.
99°C 5 min.

e. Place on ice or at 4oC for at least one min.

* Be sure that the tubes are pressed firmly in the heat block to ensure even heating.

2. A' PCR master mix:

DEPC H2o	35.5 µl
10X "215" Buffer	5.0 µl
A' primer (0.05 µg/µl)	5.0 µl
25 mM MgCl2	4.0 µl
Total	49.5 µl/rxn

Store Master mix at -20oC.
Add Taq Polymerase (2.5 units) in 0.5 µl/rxn to master mix before use.

When the RT reactions are completed, add 50 µl of A' PCR mix to each tube and cycle as indicated in the 1st DNA PCR. Follow with 2nd PCR also as described for DNA PCR.

PCR Product Analysis

Figure 1 illustrates the sequences generated by the first PCR reaction and the second, nested PCR reaction. The second PCR product is 210 base pairs and is highlighted in green bordered by the B primer in blue and the 215 primer (the wildtype in this example) in black. This sequence was generated from HXB2RT. The aa215 codon is indicated by the enlarged, black bases in bold type. Following the second PCR, 20 µl of the products are analyzed on a 3% agarose gel run in 1X TBE containing EtBr. Results are seen as bands in the lanes corresponding to reactions containing either the B/215W (sensitive) or B/215M (resistant) primers. An example of a typical gel is shown in figure 2. A mixture of sensitive and resistant virus can be

seen as bands in both lanes. However, this may also indicate that the second PCR reaction was overloaded with product from the first PCR. To eliminate this problem, the number of cycles for the first PCR can be reduced (from 30 to 15-22) and/or the dilution of products from the first PCR increased from 1:10 or 1:100 to 1:1000 or even 1:100,000 if needed to produce a single band from one or the other PCR reaction.

Application of the 215 PCR Assay to Clinical Samples

To date, over 600 patient PBMC samples and nearly 200 patient plasma (RNA) samples have been analyzed using the improved 215 PCR assay as developed by SRA Technologies, with additional samples currently being tested. The ARMS assay is now being applied to the ACTG Clinical protocol number 244 to determine its usefulness in the management of AIDS patients on antiviral therapy. Examples of these studies is presented in figures 3-4 of this report. In addition to the protocol 244, the ARMS assay for 215 has been applied to recent studies of plasma or serum viral RNA in seroconvertors (data not shown). These studies, performed for WRAIR in

Figure 1

Products of the First and Second PCRs of the ARMS Assay for aa215 of RT

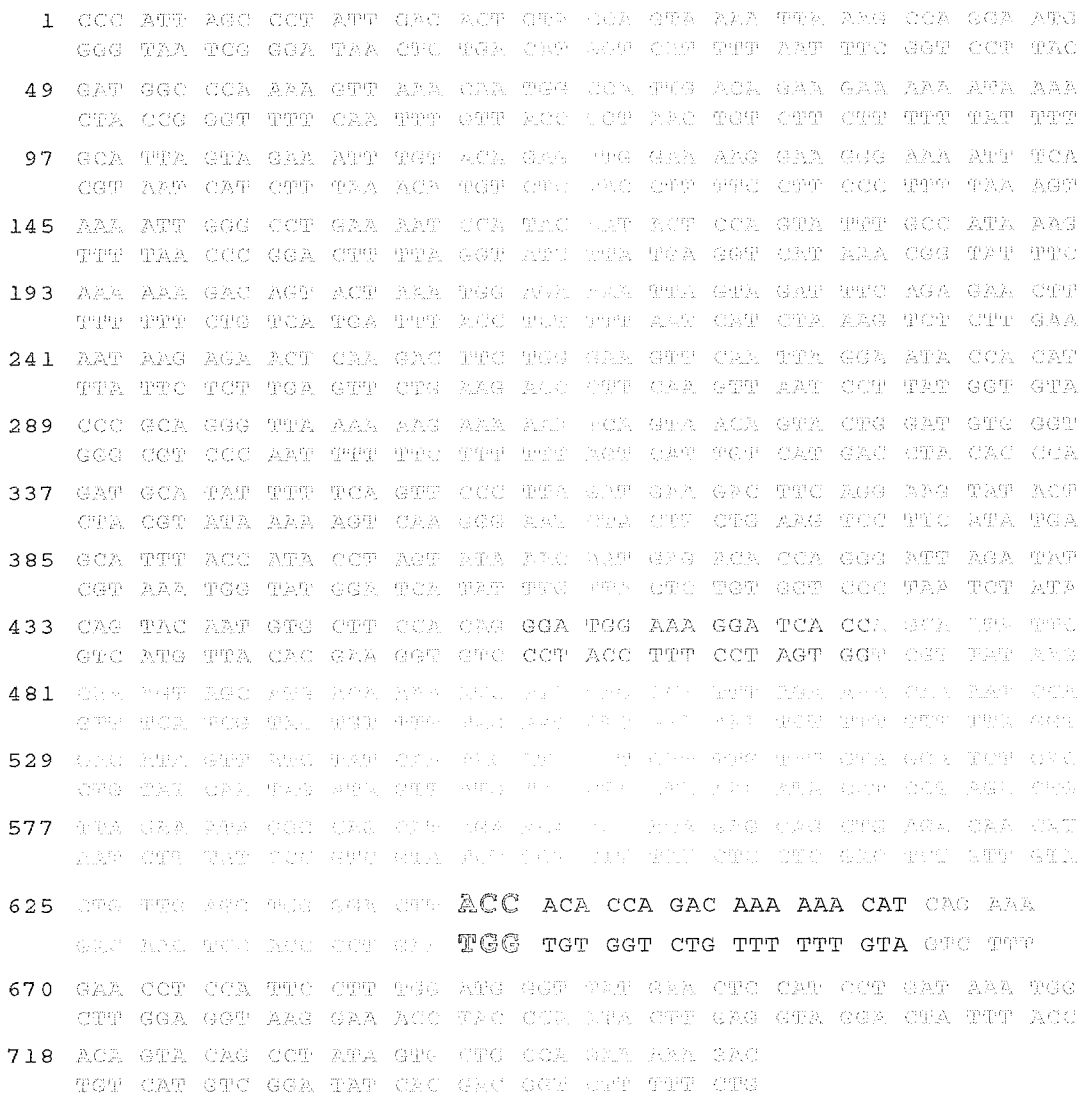
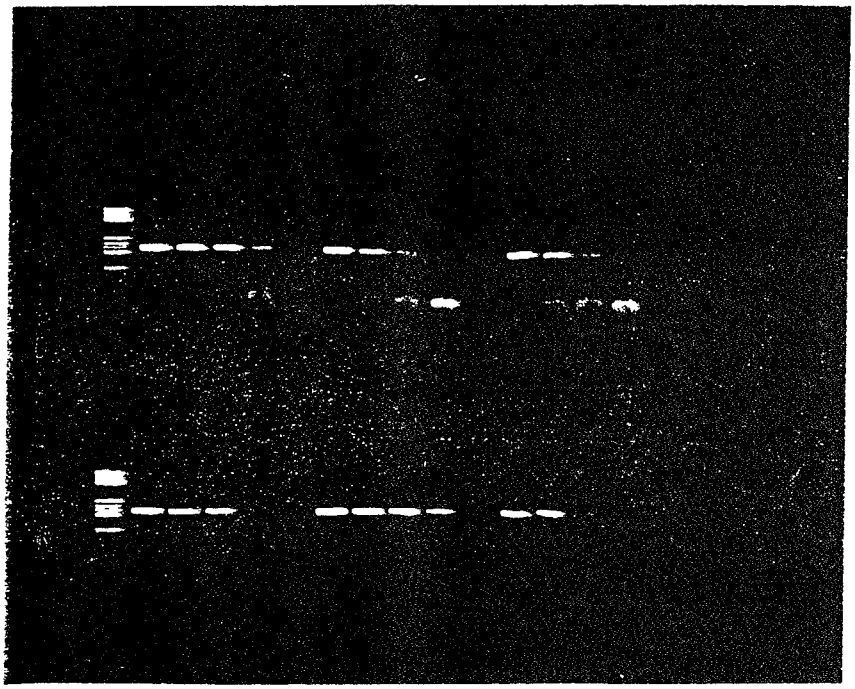


Figure 2

Typical Gel from ARMS Assay for Genotypic Resistance or Sensitivity to AZT



Lane #	Description	Result
1	Molecular Markers	
2	Specimen A 1:1000 Dilution	
3	Specimen A 1:10000 Dilution	
4	Specimen A 1:100000 Dilution	Sensitive
5	Specimen A 1:1000000 Dilution	
6	Space	
7	Specimen B 1:1000 Dilution	
8	Specimen B 1:10000 Dilution	
9	Specimen B 1:100000 Dilution	Resistant
10	Specimen B 1:1000000 Dilution	
11	Space	
12	Specimen C 1:1000 Dilution	
13	Specimen C 1:10000 Dilution	
14	Specimen C 1:100000 Dilution	Mixture
15	Specimen C 1:1000000 Dilution	

collaboration with laboratories in Swizterland, provided some of the earlist evidence for the transmission of AZT resistant HIV-1 (see abstract citation at the end of this report).

Additional ARMS Assay Development

One major limitation of the currently used agarose gel based assay is that it does not allow accurate evaluation of patient samples that contain a mixture of resistant and sensitive virus. Theoretically, the presence of a mixture of resistant and sensitive virus in a patient sample would produce PCR products from both the resistant and sensitive primer sets. These would be seen as bands appearing in both sets of lanes on a gel. Due to incomplete inhibition of primer extension from mismatched primers (such as the 215W primer hybridized to a resistant virus) however, it is possible to produce diagnostic bands of the gel from both the sensitive and resistant PCR reaction from samples that contain only one species of virus by simply overloading the second PCR reaction with product from the first (A & NE1 primer set) PCR reaction. Efforts to improve on this rather subjective procedure for analysis were not successful this past year. They included attempts to quantitate products of the second PCR using biotinylated and fluorescently tagged primers. Readout was on Molecular Dynamics FluorImager™ and both gel and capture plate formats were studied. Though product differentiation was possible, quantitation proved problematic and efforts in this direction have been curtailed.

Currently we are pursuing additional modifications to the 215 protocol with the intention of increasing the sensitivity of the assay when analyzing plasma RNA samples, and to improve the quantitative ability of the assay. A new assay procedure, available through Perkin-Elmer, the TaqManTM PCR procedure may prove more fruitful and studies, in collaboration with Perkin-Elmer's applications group will start in the new contract year.

Diagnostic DNA Sequencing

Despite the effectiveness of the 215 PCR protocol described in the previous section, it is limited in that it can only be used where the site of mutation is known, and then only when the surrounding sequence is conserved sufficiently to ensure efficient primer hybridization. For new drugs, where the site of the resistance-conferring mutation is not well characterized, or for mutations occurring in hypervariable regions, diagnostic sequencing is the only method that can provide useful genotypic information.

In FY94 SRA's sequencing protocols underwent a number of procedural modifications intended to reduce error rates frequently associated with the use of Taq DNA polymerase. When using PCR based sequencing techniques, several potential problems may arise. Several studies have examined the

error rate of *Taq* DNA polymerase when used in a PCR assay[19-21]. SRA now employs the recently introduced thermostable DNA polymerase (*Pfu* DNA polymerase, Stratagene Inc.), which is possessed of a 3'-exonuclease proofreading activity, that significantly reduces the chance of PCR induced errors in sequence determination[22]. *Pfu* polymerase has now been substituted for NEB CircumVent™ in all DNA sequencing reactions at SRA. Our latest procedure for sequencing of a portion HIV's RT gene is described below.

Sequecing Step-by-Step Procedure

A. Template Preparation

It is recommended that PCR products be generated by a method called "Ampliwax Hot Start". Better sequencing results have been obtained using "Hot Start" PCR products because fewer non-specific products are generated.

1. Purify template by placing into the retentate cup of a Microcon 100 with 300 µl TE buffer.

2. Microcentrifuge at 3,000 RPM's for 9 minutes.

3. Empty the waste in the centrifuge tube and repeat step #2 two more times, each time washing with 400 µl of TE buffer.

4. After the last wash, remove the cap and place retentate cup into a clean catch tube and turn upside down, so that the sample reservoir is inside the centrifuge tube. Centrifuge at 1,000 RPM's for 5 min.

B. Sequencing Reaction

1. Label four 0.5 ml centrifuge tubes A,G,C and T. Place 3 µl of ddATP termination mix into the tube labeled A, and do the same for G,C and T tubes. Store on ice.

2. In a separate 0.5 ml centrifuge tube, combine the following:

1/20 volume of purified PCR product
2 pmole Primer
4 µl 10x sequencing buffer
1 µl α ^{33}P dATP (10 µCi)
1 µl Exo- PFU polymerase (2.5 U)
Bring volume to 26 µl with ultra pure water
4 µl DMSO

---

final volume = 30 µl

Mix well by pipetting and a brief spin in the centrifuge. Keep on ice.

3. Immediately aliquot 7 µl of the reaction mixture from step #2 into each of the 4 termination tubes containing 3 µl of their appropriate ddNTP. Mix thoroughly, making sure reaction mix and dideoxynucleotide mix are at the bottom of the tube.

4. Overlay the reaction with 15 µl mineral oil. Briefly centrifuge.

5. Cycle reactions as follows:

   Denature at 95°C for 5 minutes
   95°C for 30 s
   60°C for 30 s — 30 cycles
   72°C for 1 min.
   Hold at 72°C

   ***Note:** Reactions should not be held for more than 5 minutes at 72°C, degradation can occur. The cycling takes approximately 1 1/2 - 2 hours.

6. At the end of the cycling procedure, add 5 µl of stop solution to each reaction tube and immediately place on ice.

7. When ready to load gel, denature samples at 75-80°C for 5 minutes, place on ice and load 3 µl on gel. Otherwise, samples can be stored at -70°C until ready for use.

Sequence Data Analysis

Currently, DNA sequence information is acquired with a Howtek Scanmaster 3+ flat bed scanner used to digitalize the entire 35X43 cm film of the sequencing gel. Using input data from digitized films the Millipore/BioImage analysis software SRA currently runs on a Sun Sparc 10 workstation can automatically define the sequencing reaction lanes and perform automatic base calling using neural network algorithms for increased accuracy. These advanced algorithms can be "trained" to improve base-calling accuracy the more they are used. In addition, the software allows quantitation of band intensity from the sequencing gel, facilitating determination of mixed population at a particular base location based in the sequencing gel information.

This system is currently connected to our LAN (described in the reports

section) and can access other computers both in-house and through the Internet. The system can also interact with other molecular biology software packages, such as the DNAStar Lasergene system currently running on a Mac IIci with Genbank on CD-Rom. Custom filters have been provided to directly access the Mac format DNAStar files for use on the SUN system. These capabilities will provide easy access to the sequence data generated in our laboratories, and the information generated can easily be included in reports requested by NIAID.

Application of Diagnostic DNA Sequencing to Clinical Samples

We continue to apply this technology to confirm the results of the 215 PCR assay for the presence of mutations conferring AZT resistance. For example, sequencing was by employed by SRA to provide confirmatory evidence for the presence of drug resistance virus in seroconverters. Moreover, sequencing has been applied to the evaluation of biologically cloned, drug resistant viruses prepared by NCI in collaboration with WRAIR. Results of these sequencing analyses can be seen in tables 1-7. Each resistant mutant sequence was independently aligned with a standard published sequence for HXB2-RT and/or the parental wild type HIV-1_{IIIb}. Sequence differences are noted in the figures along with amino acid positions and changes if any. Positions containing more than one amino acid means that there was a mixed base pair present. The data represent any changes in the first 750 bp or 250 aa of the HIV-RT sequence shown in figure 1.

Table 1

Amino Acid #	Virus Sequence HXB2RT	IIIB	DRUG A RESIST
106	GTA (Val)	GTA (Val)	ATA (Ile)
108	GTA (Val)	GTA (Val)	GTA or ATA (Val) or (Ile)
136	AAC (Asn)	AAC or AAT (Asn) or (Asn)	AAT (Asn)
200	ACA (Thr)	ACA (Thr)	ACT (Thr)
214	CTT (Leu)	CTT (Leu)	TTT (Phe)

Table 2

Amino Acid #	Virus Sequenced HXB2RT	IIIB	DRUG B RESIST
90	GTT (Val)	GTT (Val)	GTT or ATT (Val) or (Ile)
136	AAC or AAT (Asn) or (Asn)	AAC or AAT (Asn) or (Asn)	AAC or AAT (Asn) or (Asn)
139	ACA (Thr)	ACA (Thr)	ACA or ATA (Thr) or (Ile)
214	CTT (Leu)	CTT (Leu)	TTT (Phe)
225	CCT (Pro)	CCT (Pro)	CCT or TCT (Pro) or (Ser)

Table 3

Amino Acid #	Virus Sequenced HXB2RT	IIIB	DRUG C RESIST	DRUG D RESIST
26	TTG (Leu)	TTG (Leu)	TTA (Leu)	TTG (Leu)
40	GAG (Glu)	GAG (Glu)	GAA (Glu)	GAG (Glu)
108	GTA (Val)	GTA (Val)	ATA (Ile)	ATA (Ile)
136	AAC (Asn)	AAC or AAT (Asn)	AAT (Asn)	AAC (Asn)
160	TTC (Phe)	TTC (Phe)	TTC (Phe)	TTT (Phe)
214	CTT (Leu)	CTT (Leu)	TTT (Phe)	TTT (Phe)

Table 4

Amino Acid #	Virus Sequenced IIIB	DRUG E RES.	DRUG F RES.	DRUG G RES.
21	GTT (Val)			GTT or ATT (Val) or (Ile)
40	GAG (Glu)	GAA (Glu)		
90	GTT (Val)	ATT (Ile)		GTT or ATT (Val) or (Ile)
103	AAA (Lys)	GAA (Glu)		
106	GTA (Val)	GTA or ATA (Val) or (Ile)		
136	AAC or AAT (Asn) or (Asn)	AAT (Asn)	AAC or AAT (Asn) or (Asn)	AAC or AAT (Asn) or (Asn)
170	CCT (Pro)			CCT or CTT (Pro) or (Leu)

Table 5

Sample	Amino Acid # 18	40	62	98	100	108	136	150	160	180	181	192	205	214	229
HIVHXB2	GGC (Gly)	GAG (Glu)	GCC (Ala)	GCA (Ala)	TTA (Leu)	GTA (Val)	AAC (Asn)	CCA (Pro)	TTC (Phe)		TAT (Tyr)	GAC (Asp)	CTG (Leu)	CTT (Leu)	TGG (Trp)
DRUG M RESIST.			GCC (Ala) GTC (Val)	GGA (Gly) GCA (Ala)		ATA (Ile)	AAT (Asn)	-						CTT (Leu) TTT (Phe)	
DRUG N RESIST.		GAA (Glu)			ATA (Ile)								CTA (Leu)		
DRUG O RESIST.						ATA (Ile)			TTT (Phe)					TTT (Phe) CTT (Leu)	TGG (Trp) TTG (Leu)
DRUG P RESIST.	GGC (Gly) GGT (Gly)										TGT (Cys)				
DRUG Q RESIST.		GAA (Glu) GAG (Glu)						-			TGT (Cys)	GAC (Asp) GAT (Asp)			
IIIB							AAC (Asn) AAT (Asn)	-							

Table 6

Amino Acid #	Virus Sequenced HXB2RT	IIIB	DRUG H RES	DRUG I RES	DRUG J RES
40	GAG (Glu)	GAG (Glu)	GAG (Glu)	GAG (Glu)	GAA (Glu)
108	GTA (Val)	GTA (Val)	GTA (Val)	ATA (Ile)	GTA (Val)
136	AAC (Asp)	AAC or AAT (Asp) or (Asp)	AAC (Asp)	AAC (Asp)	AAC (Asp)
160	TTC (Phe)	TTC (Phe)	TTC (Phe)	TTT (Phe)	TTC (Phe)
181	TAT (Tyr)	TAT (Tyr)	TGT (Cys)	TAT (Tyr)	TGT (Cys)
192	GAC (Asp)	GAC (Asp)	GAC or GAT (Asp) or (Asp)	GAC (Asp)	GAC (Asp)
214	CTT (Leu)	CTT (Leu)	CTT (Leu)	TTT (Phe)	CTT (Leu)

Table 7

Amino Acid #	Virus Sequenced IIIB	DRUG K RES	DRUG L RES
40	GAG (Glu)	GAA (Glu)	
108	GTA (Val)	ATA (Ile)	
136	AAC or AAT (Asn) or (Asn)	AAT (Asn)	AAC (Asn)
181	TAT (Tyr)		TGT (Cys)
214	CTT (Leu)	TTT (Phe)	
224	GAA (Glu)		GAA or AAA (Glu) OR (Lys)
240	ACA (Thr)	ATA (Ile)	

2. Cellular Phenotype Working Group

The Cellular Phenotype Working Group worked in three major areas during the current contract year. These included; 1) developing and validating phenotypic assays to support evaluation of neutralizing antibodies in vaccinees, 2) developing and implementing *in vitro* systems to evaluate antiviral genes for the treatment of HIV disease and 3) performing in vitro antiviral drug studies and other phenotypic analyses of patient isolates in support of clinical HIV chemotherapy. The progress made during the past year in each of these areas is reviewed below.

Development and Optimization of Virus Titration and Neutralization Assays

Virus neutralization assays may be employed to identify and differentiate virus, as well as to determine the host immune responsiveness to a specific viral infection and/or vaccination with various viral protein(s). Although identification of serum antibodies which inhibit viral infection *in vitro* may be a useful marker of protective immunity for some viruses *in vivo*[23], the significance of neutralizing antibodies in influencing clinical outcome in HIV infected individuals is not well understood.[24-30] Currently, there are a number of assays being used to evaluate the effect of antibody on HIV replication. Originally, most studies utilized immortalized cells (*e.g.*, H-9, MT-2, etc) that exhibited susceptibility to one or more laboratory strains of HIV (IIIb, RF, MN, etc). Susceptibility was usually evaluated by the production of viral markers and/or the induction of cytopathic effects (CPE).[31-35] However, most field isolates of HIV (*i.e.*, low passaged, patient isolates) infect immortalized cells with very low efficiency, thus these assays are of limited value in assessing the neutralizing antibody titers of a patients sera to clinical isolates.

Several Projects were completed this Contract year that were started in the last Contract year. The projects fall into two categories:

1. Testing of vaccinee sera to determine neutralizing potential and specificity, if any, of candidate vaccines for a panel of laboratory virus strains and selected virus isolates, and

2. Screening and titration of naturally-occuring (infected patients) immune sera against viral isolates in an attempt to determine if previously determined genotypes correlate with apparent serotypes.

The general strategy for both categories has been to screen sera against a typical laboratory strains and several clinical isolates; then to carefully characterize the "neutralizing" sera using serum titration in an attempt to rank the sera. The panels of sera tested in the first category were recieved through Dr. John Mascola from the AIDS Vaccine Evaluation Group (AVEG) and a private biotech firm (Chiron). A summary of the screening from the

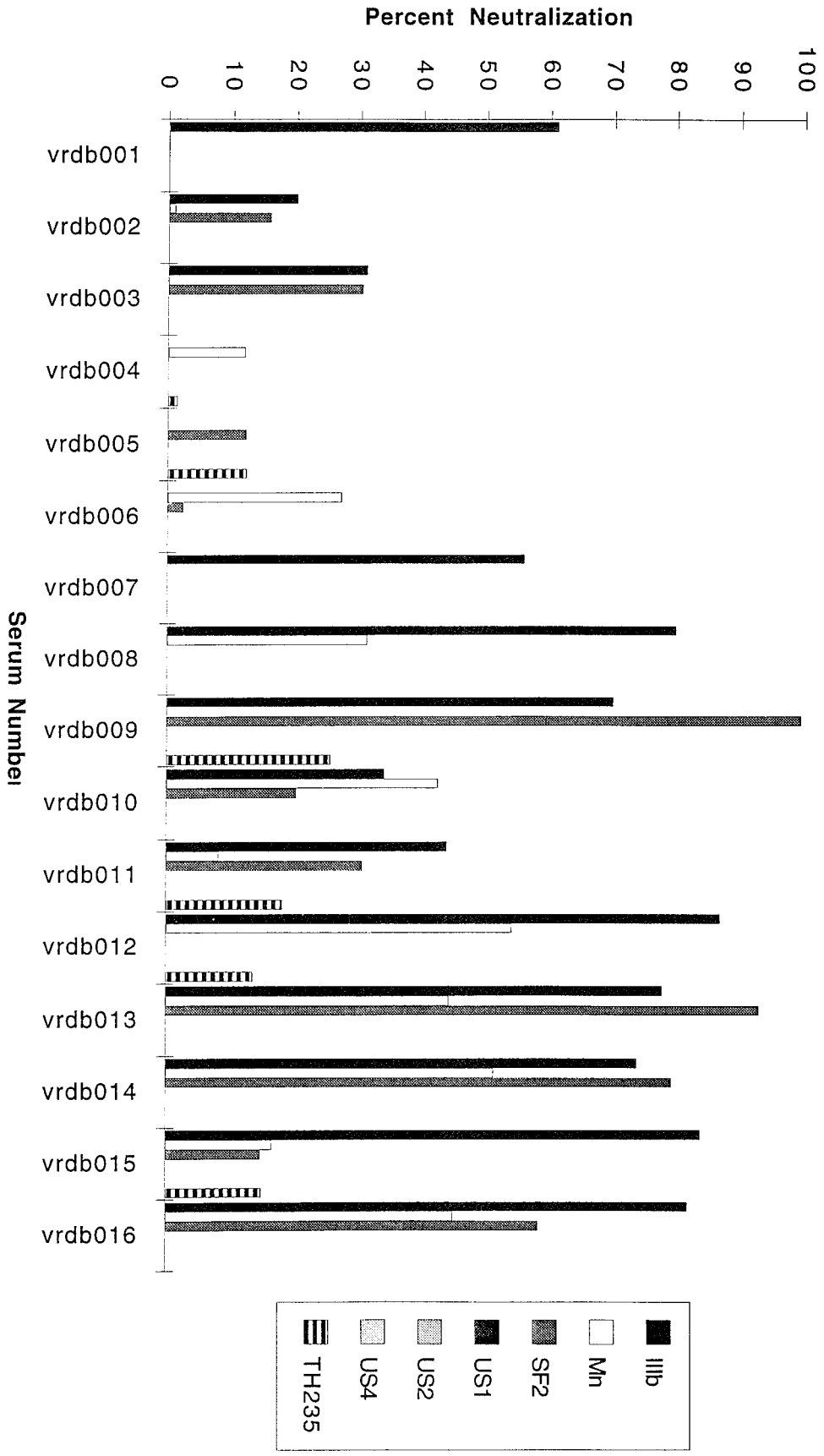


Figure 3 Neut. Screen-AVEG Panel

Figure 4 Neut. Screen-AVEG Panel :

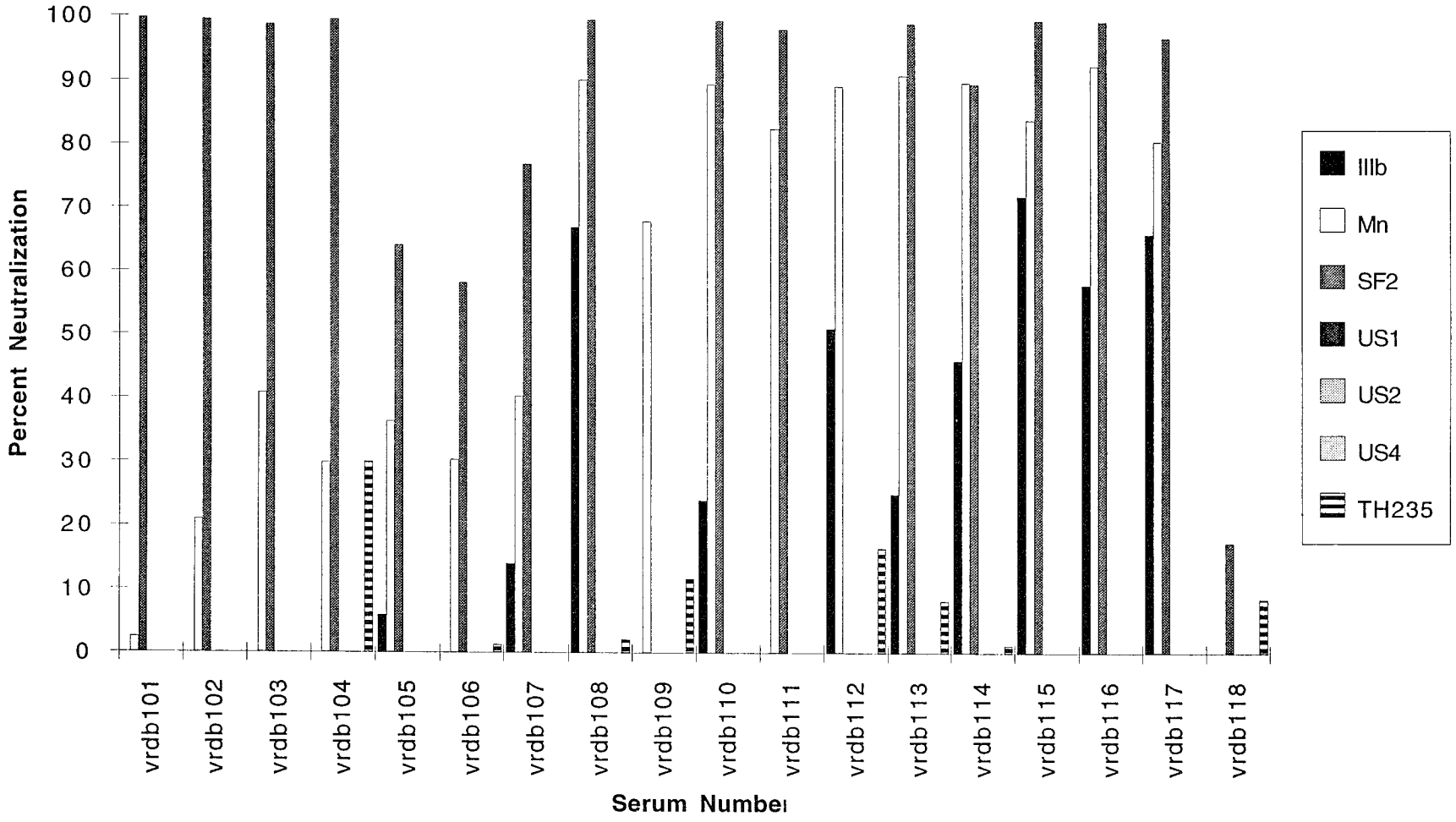


Figure 5 Neut. Screening-AVEG Panel

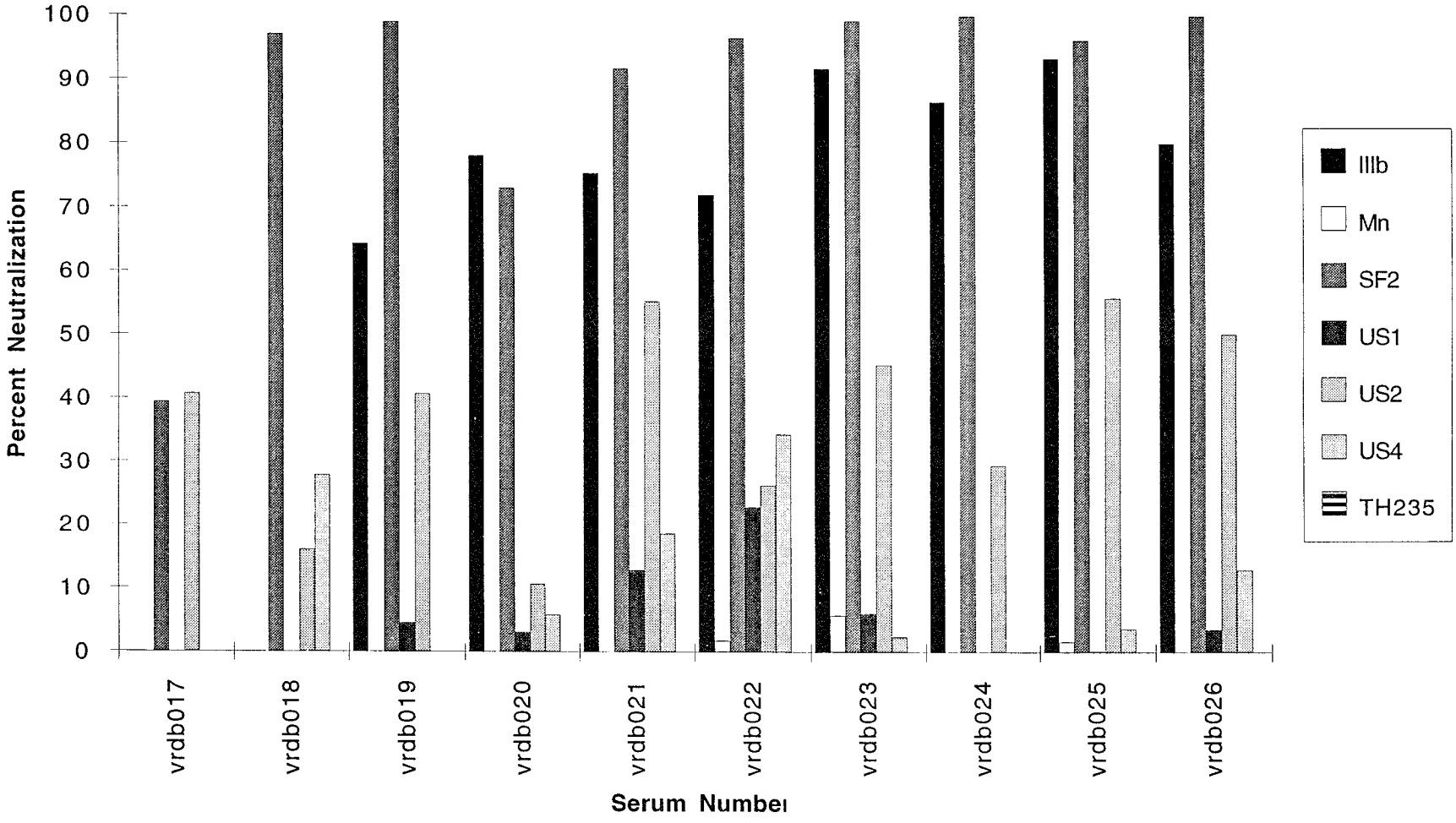


Figure 6 Neut. Screen-AVEG Panel

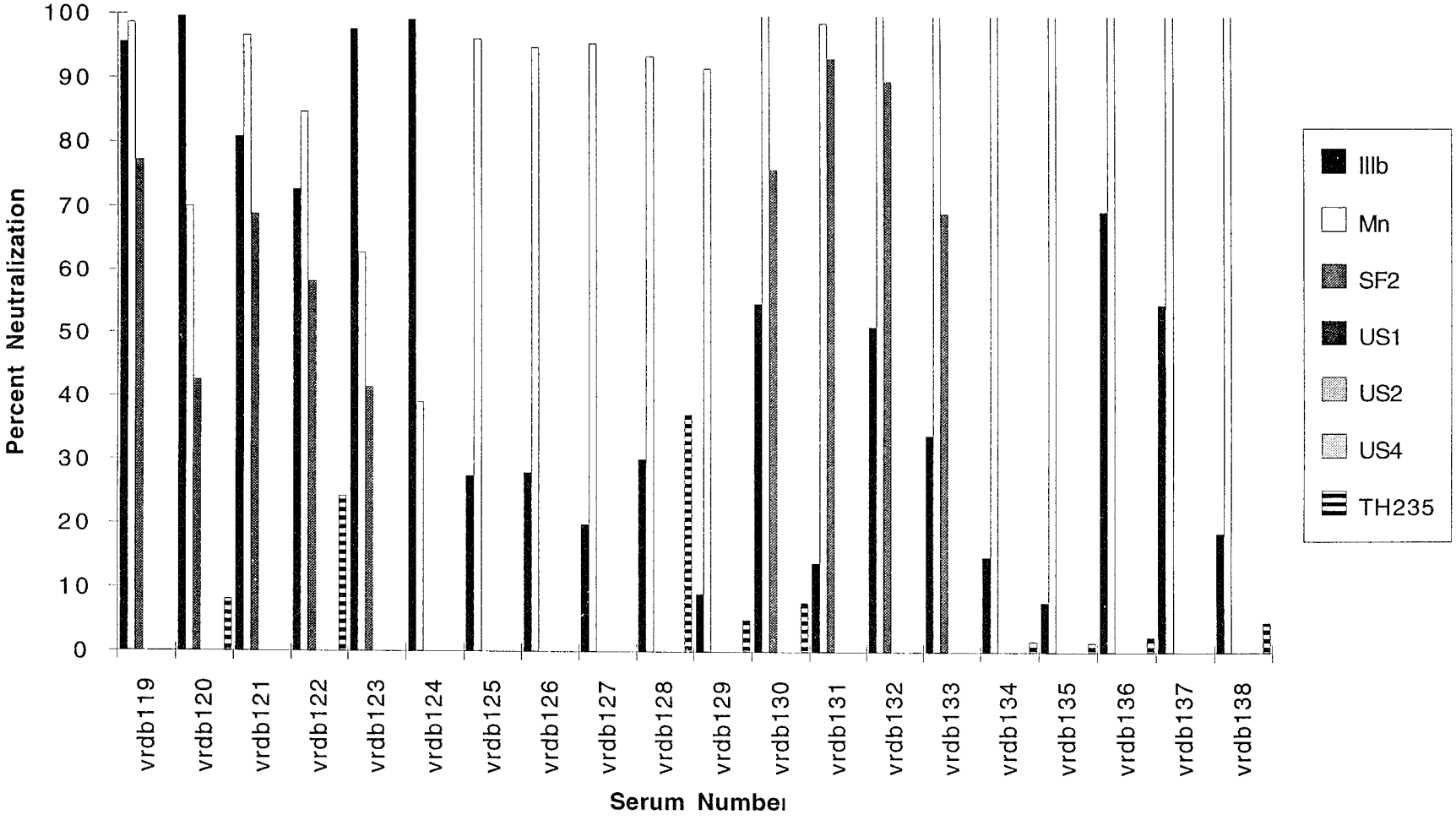


Figure 7 Neut. Screen-lab Strains & Primary Isolat

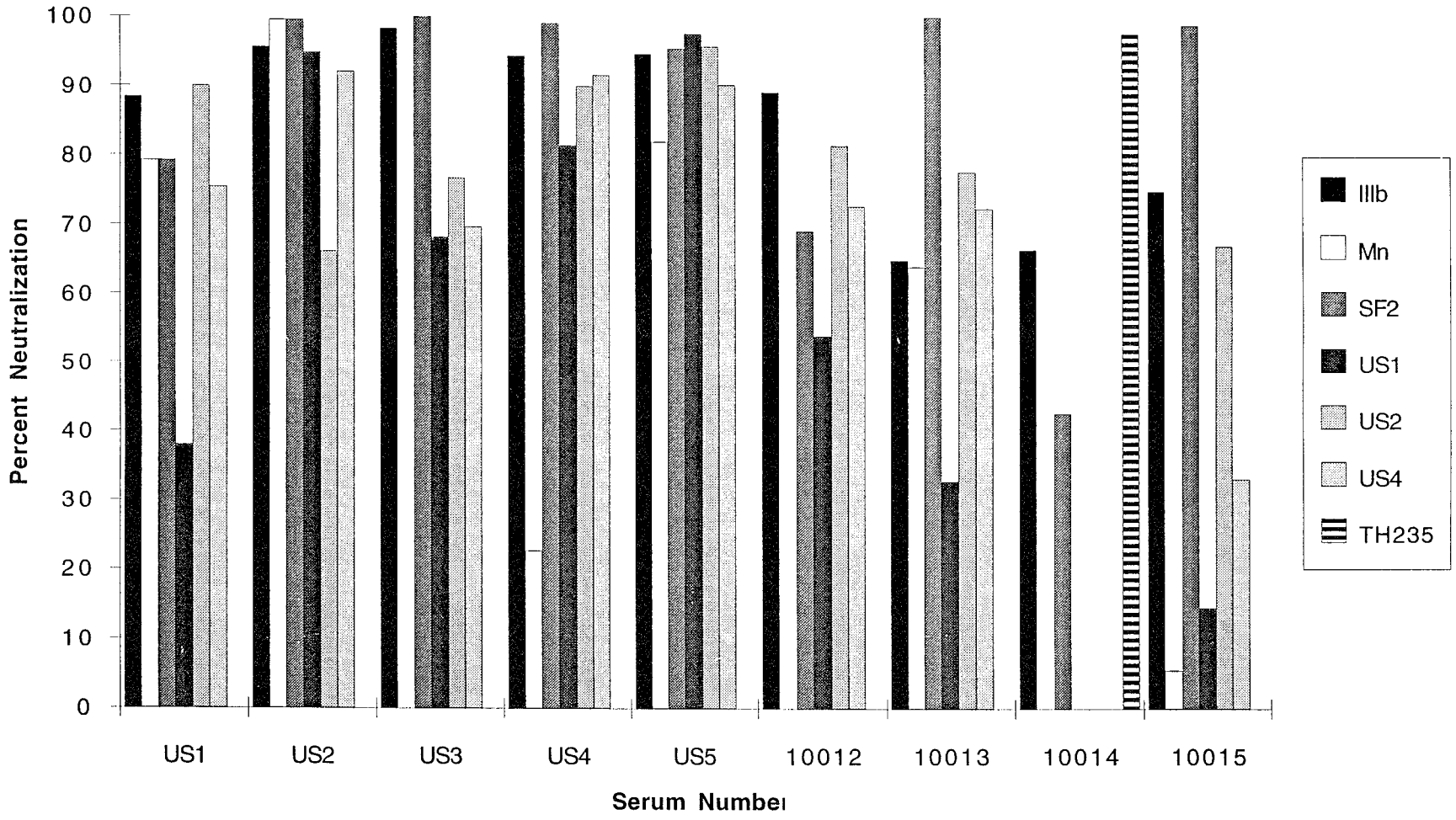


Table 8 Neutralization of Lab Strains of HIV By Patient Sera

	Mn		SF2		IIIb	
SERUM ID	% Neut	Titer	% Neut	Titer	% Neut	Titer
US3	99	14000	99	3500	94	1400
US5	99	26000	99	6200	96	210
Seroneg	<50	<10	<50	<10	<50	<10
VRDB008	<50	<10	<50	<10	86	90
VRDB018	94	110	<50	<10	<50	NT
VRDB103	80	70	90	150	<50	<10
VRDB117	95	12700	96	1500	78	50
VRDB118	<50	<10	<50	<10	<50	<10

* NT: Not Titered

Figure 8 Neut. Screen-International Isolat

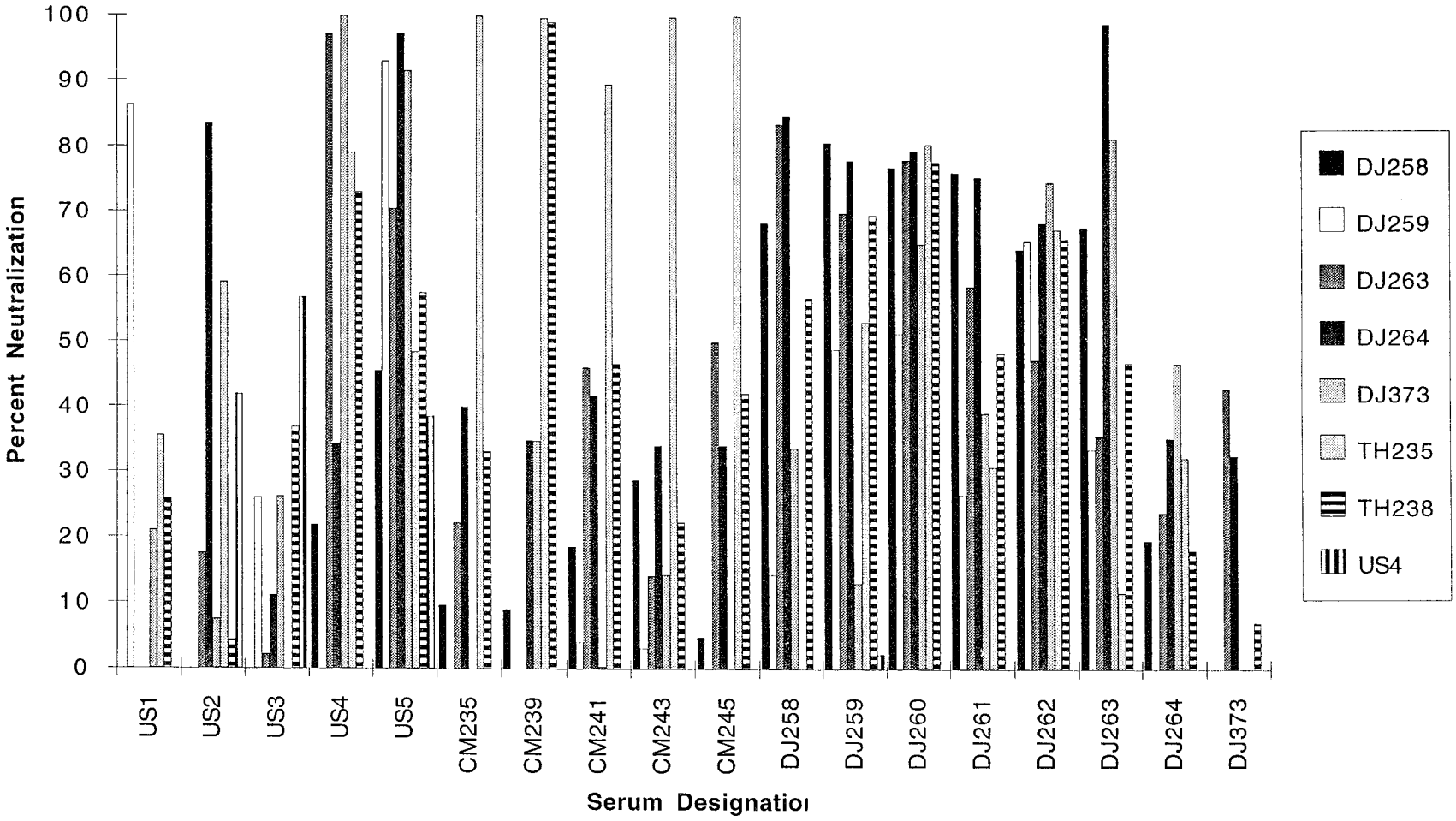


Table 9 SUMMARY OF WHO SERUM SCREENING RESULTS

VIRUS

	Group A.				Group B				Group C		Group D		Group E	
	RW09		UG37		BR20		TH14		BR25		UG24		TH22	
SERUM	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index
RW09	26088	1.50	5418	1.25	24447	2.22	7235	3.19	2858	3.16	318000	2.66	3170	2.77
UG37	18581	2.11	3038	2.24	8408	6.46	3385	6.83	167	53.98	148914	5.68	1034	8.51
BR20	11813	3.31	5465	1.24	20743	2.62	5932	3.90	3885	2.32	432200	1.96	2758	3.19
TH14	21867	1.79	5281	1.29	27202	2.00	7155	3.23	3095	2.92	229200	3.69	4301	2.05
BR25	25625	1.53	4857	1.40	26348	2.06	6269	3.69	3392	2.66	388800	2.18	3601	2.44
TH22	14074	2.78	6134	1.11	25680	2.12	11859	1.95	4856	1.86	200781	4.21	3129	2.81
UG24	23074	1.70	3872	1.75	18243	2.98	3347	6.90	1826	4.94	101886	8.31	1641	5.36
FDA2	9892	3.96	127	53.55	627	86.59	418	55.28	347	25.99	356400	2.37	1033	8.52
NHS	39130	1.00	6790	1.00	54324	1.00	23106	1.00	9028	1.00	846200	1.00	8797	1.00

Table 10 SUMMARY OF WHO VIRUS REDUCTION ASSAY

TCID50 (STANDARD DEVIATION) IN THE PRESENCE OF:

	NHS(1)		NHS(2)		NHS (mean)		10013		9170	
VIRUS	TCID50	STDEV	TCID50	STDEV	TCID50	STDEV	TCID50	STDEV	TCID50	STDEV
TH235	127	27	143	9	135	6	29	3	96	6
US1	1460	43	510	21	808	22	1490	44	33	3

log[TCID50(NHS)/TCID50(test)]

VIRUS	NHS(1)	NHS(2)	NHS (mean)	TH243	US2
TH235	0.03	-0.03	0.00	0.67	0.15
US1	-0.26	0.20	0.00	-1.04	0.62

AVEG can be seen in figures 3-7. The data presented indicate that sera from vaccinees--immunized with glycoproteins from laboratory strains--are able to neutralize laboratory strains, but not primary isolates. The results of the screening were confirmed by further titrating some of the sera that showed significant neutralization, *i.e.*, > 75% neutralization of the virus. Table 8 summarizes representative data from serum titration experiments. In addition to non-neutralizing sera, sera were found that were narrowly neutralizing--*e.g.*, VRDB008 and VRDB018--whereas other sera were broadly neutralizing--*e.g.*, VRDB117.

The sera of the second category were recieved directly from MMCARR collaborators of Dr. Mascola--International Isolates--or from the World Health Organization--WHO. Figure 8 summarizes representative data from the International Isolate panels. In these, as previously reported by Dr. Mascola, sera seem to neutralize better within their genotypic clade.

The experiments using sera recieved from the WHO are part of another effort to serotype HIV. The data of Table 9 represent this laboratory's part in comparing two different typing methods: Serum Titration and Infectivity Reduction Assays--IRA. The data of Table 9 were compared with other data obtained in a collaborating lab (Dr. Peter Nara) and although there is good agreement between the results of the two methods, the IRA results are more ameable to statistical analysis and requires less work; representitive data from IRA titrations are presented in Table 10.

Virus Expansions and Evaluation of Cytopathic Effects

A substantial part of this contract's resources are alloted to the expansion and titration of viral stocks. In addition, investigations of the biological characteristics of viral isolates represents an important part of all clinical protocols supported by this contract. Table 11 summarizes the viral expansions that were performed in FY94. In addition to viral expansions and titrations the laboratory performed approximately 576 evaluations of the syncytial inducing capacity of isolates cultured from patients on the RV43 protocol for antiviral drug resistance.

Research and Development

A significant portion of this laboratories support of the 490 contract has been Vaccine Development. The neutralization assays described above have been exclusively used at SRA. In collaboration with Dr. J. Mascola, a series of experiments have been conducted in the FY/94 contract year to better understand the results of neutralization assays; we have examined the kinetics of replication of several viruses, the effect and kinetics of neutralization prior to infection, the effect of neutralization concomitant with infection and neutralization after virus binding to cells was thought to occur. It is well known that different viruses replicate at different rates. We

Table 11 EXPANSION DATA

Specimen				SOURCE INFORMATION					Harvest Data		Titration Information			
Spec_id	Alt_id	Harvest Date	Setup Date	Spec_id	Harvest Date	Spec Type	Hvst Day	p24 Conc. (ng/ml)	Hvst Day	[p24] ng/ml	Cells per Well	Leukopak Used	Day 4 TCID50	Day 7 TCID50
1160	Brazil	3/4/94	2/25/94	1160	2/18/94	VS	8	212	7	693	200K	22981	43238	24834
1161	Brazil	8/9/94	8/1/94	1161	7/20/94	VS	4	37	8	615	200K	26303		294
1161	Brazil	8/11/94	8/1/94	1161	7/20/94	VS	4	37	10	149	200K	26303		512
1162	Brazil	3/4/94	2/25/94	1162	2/18/94	VS	8	313	7	1130	200K	22981	228210	75281
3074	Brazil	8/9/94	8/1/94	3074	7/17/94	VS	7	9.4	8	167	200K	26303		675
3074	Brazil	8/11/94	8/1/94	3074	7/17/94	VS	7	9.4	10	90	200K	26303		2,702
3899	Brazil	3/15/94	3/8/94	3899	2/2/94	VS	8	115	7	211				6208
3906	Brazil	1/14/94	1/7/94	3906	12/23/93	VS	7	134	7	749	200K	28456	42238	102127
3906	Brazil	1/17/94	1/7/94	3906	12/23/93	VS	7	134	10	310	200K	28456	2702	14263
3907	Brazil	1/18/94	1/14/94	3907	12/23/93	VS	7	67	4	202				131072
3907	Brazil	1/20/94	1/14/94	3907	12/23/93	VS	7	67	6	194	200K	28456	4705	32768
3909	Brazil	1/14/94	1/7/94	3909	12/23/93	VS	7	69	7	310	200K	28456	32768	262144
3909	Brazil	1/17/94	1/7/94	3909	12/23/93	VS	7	69	10	310	200K	28456	1552	14263
4969		5/2/94	4/25/94	4969.3	4/30/92	VS	10	93.2	7	6.6	200K	31166		4705
4971.5		11/2/93	10/24/93	4971.5		VS	7	479	7	1310	200K	23278	4705	43238
4971.5		11/5/93	10/24/93	4971.5		VS	7	479	10	350	200K	23278	2702	75281
4971.5		3/21/94							7					
4974		4/15/94	4/8/94	4974	4/5/94	VS	7	8	7	65.7	200K	25860		388
4981		4/15/94	4/8/94	4981	4/27/92	VS	7	341	7	55.6	200K	30702	2048	4705
5204		2/14/94	2/7/94						7	230	200K	28624	24833	75281
8871		4/15/94	4/8/94	8871	4/5/94	VS	7	19.7	7	60	200K	30702	294	1176
9310		2/5/94	1/29/94	9310	1/23/94	VS	14	37	7	119	200K	28624	3565	14263
9310		2/8/94	1/29/94	9310	1/23/94	VS	14	37	10	94	200K	28624	18820	6208
9667		6/3/94	5/30/94	9667.2	8/31/92	VS	11		4	51.8				
9667		6/6/94	5/30/94	9667.2	8/31/92	VS	11		7	105	200K	25860		43237
9881	US1	1/25/94	1/18/94	9881					7	642	200K	25860	21619	1383604
9881	US1	3/21/94	3/14/94	9881	1/25/94	VS	NA	642	7	133	200K	30702	32768	75281
9882	US2	1/25/94	1/18/94	9882					7	317	200K	28456	6208	18820
9882	US2	3/22/94	3/16/94	9882	1/25/94	VS	7	317	6	305	200K	30702	32768	172950
24346		10/15/93	10/8/93	24346				23	7	45	200K	31211		2048
24346		10/18/93	10/8/93	24346				23	10		200K	31211		24833
24620		10/28/93	10/21/93	24620	11/8/92	VS	10	77	7	205	200K	28456	42	128
24620		11/1/93	10/21/93	24620	11/8/92	VS	10	77	10	279	200K	28456	891	24833
24777	SF2(p)	11/5/93	10/28/93	24777				122	7	120	200K	28456	388	2048
24777	SF2(p)	11/8/93	10/28/93	24777				122	10	291	200K	28456	294	891
0370	Zambi		4/11/94	0370	4/4/94	VS	7	3.7						
0370	Zambi	6/20/94	6/13/94	0370	6/3/94	SU	7	49.7	7	20.3				
0370	Zambi	6/23/94	6/13/94	0370	6/3/94	SU	7	49.7	10	77.6				
0557	Zambi	4/4/94	3/28/94	0557	8/7/92	VS	7	138	7	67.9	200K	25860		24883

Table 11 EXPANSION DATA

Specimen				SOURCE INFORMATION					Harvest Data		Titration Information			
Spec_id	Alt_id	Harvest Date	Setup Date	Spec_id	Harvest Date	Spec Type	Hvst Day	p24 Conc. (ng/ml)	Hvst Day	[p24] ng/ml	Cells per Well	Leukopak Used	Day 4 TCID50	Day 7 TCID50
IIIB.2	IIIB	11/2/93	10/24/93	IIIB.2	7/9/92	VS	7	1280	7	1020	200K	23278	4705	32768
IIIB.2	IIIB	11/5/93	10/24/93	IIIB.2	7/9/92	VS	7	1280	10	412	200K	23278	4705	131072
IIIB.2	IIIB	8/29/94	8/23/94	IIIB		VS			6	134				
MN.2	MN	11/2/93	10/24/93	MN.2	7/9/92	VS	7	480	7	133				
MN.2	MN	11/5/93	10/24/93	MN.2	7/9/92	VS	7	480	10	152	50K	26499	294	362
MN.2	MN	11/19/93	11/12/93	MN	11/2/93	VS	7	133	7	212	200K	31211		3566
MN.2	MN	11/22/93	11/12/93	MN	11/2/93	VS	7	133	10	293	200K	31211		24834
MN.2	MN	2/7/94	1/31/94	MN	11/19/93	VS	7	212	7	419	200K	28624	6208	24833
MN.2	MN	8/29/94	8/23/94	MN/H9	5/18/92	VS	7		6	478				

confirmed the many previous observations of others and characterized 4 specific viruses. Characterization of different replication rates is directly relevant to the interpretation of subsequent neutralization experiments; *e.g.*, we need to know if the apparent decrease in replication of the virus in the presence of "neutralizing" antisera is merely an artifact of replication kinetics or true neutralization. The four viruses--two laboratory-adapted strains and two primary isolates--show wide differences in infection rates.
We have also found that laboratory viruses are neutralized more easily (or more quickly) than primary isolates prior to infection. In two separate experiments, virus and antisera were incubated for up to 16 hours prior to the addition of cells. After a standard infection period, the virus and antisera were removed and then viral replication allowed to proceed. The results of each experiment were the same: No statistically significant neutralization of primary isolates by the antisera used was measured in contrast with marked and time dependant neutralization of laboratory-adapted viruses.

In contrast to these results, both laboratory-adapted and primary isolates appear to be neutralized in a time-dependant manner. In several experiments, virus and cells were incubated for up to 16 hours prior to the addition of virus. After a standard "neutralization" period (1-2 h), the virus and antisera were removed and then viral replication allowed to proceed. The results of each experiment were the same: Statistically significant, time dependant neutralization of both primary and laboratory-adapted isolates by the antisera used was measured. The rates of neutralization differed for each virus and did not group according to source; *i.e.*, laboratory isolates are not neutralized faster or slower than primary isolates.

The results described suggest that laboratory-adapted viruses may be neutralized by an additional mechanism to that of primary isolates. The experiments performed to date are preliminary and have certain inherent limitations: Small sample sizes of both virus and antisera, suboptimal experimental control, and--in the earlier experiments--lack of the ability to statistical analyze the data because of experimental design. Using the virus infectivity reduction assay (described above) and newly available statistical evaluation software, we will be able to overcome these limitations. Confirmation and elaboration of the kinetic results are actively being pursued in this laboratory.

Evaluation of Antiviral Gene Constructs

Gene therapy for immunological disorders, cancers and a variety of infectious diseases is quickly becoming a reality. This approach has been expanded from "simple" gene replacement or augmentation therapy to correct a genetic defect (as in the case of adenosine deaminase, ADA, deficiency [36,37] to new genetic treatments for cancers[38] and infectious diseases such as AIDS.[39,40] There have been numerous

proposals for the treatment of HIV infections using antisense genes [41-45] and genes containing catalytic RNAs (ribozymes).[46,47] *In vitro* interference with viral replication has been accomplished by targeting gene constructs to viral structural proteins[48-52], components of HIV's regulatory circuits[53-55] and the virus receptor, CD4.[56] The number of antiviral gene constructs available for testing appears to be multiplying exponentially.

Preliminary *in vitro* evaluation of these therapies has been accomplished, for the most part, in artificial systems sometimes employing biochemical endpoints or in well established cell lines using laboratory strains of HIV. Little is known about the efficacy of such treatments for primary isolates of HIV in normal human peripheral blood mononuclear cells (PBMC) and there are no published reports of quantitative determinations of putative antiviral gene effects on primary isolate-induced cytopathogenesis. Moreover, the impact of these constructs on the differentiation and ultimate immune function of human bone marrow derived hematopoietic stem cells, the apparent conveyance of choice for some gene constructs, is little understood. Finally, there still is no *in vitro* testing system available to bridge the gap between preclinical *in vitro* analyses and animal model systems such as the SIV model in macques.

In support of a WRAIR's gene therapy research and development, SRA was requested to develop *in vitro* assay systems to assess the efficacy of antiviral gene constructs against low passage, clinical isolates of HIV. Initial studies were to involve the use of syncytial-inducing isolates of HIV in established cell lines previously transfected with antiviral genes. This was to be followed by similar studies in PBMCs that would permit evaluation of a broader range of clinical isolates or, eventually, a prospective patient's own cells. The cell line chosen for the preliminary studies was MT-2, a line that is productively infected with HTLV-1, but is sensitive to infection by ≈35% of patient isolates. The initial studies conducted with these cells failed in the last fiscal year suggesting that production of HTLV-1 may have blocked the action(s) of the antiviral genes understudy. This virus could concievably interfer with expression, regulation or activities of the antivirals. New studies were conducted in FY94 using the A3.01 and SupT-1 cell lines, both of which are free of HTLV-1. Tables 12, 13, 14 and 15 illustrate the antiviral effects of a number of antiviral gene constructs using both reverse transcriptase and p24 endpoints in SupT-1 or A3.01 cell lines. Spurred by these sucesses we have begun to evaluate the possible use of purified CD4+ PBMCs in gene therapy by studying the susceptibility of PBMCs, stimulated with anti-CD28 and anti-CD3 antibodies, to virus infection. Our intention is to use cells, stimulated with these agents, and grown for extended periods *ex vivo*, as vehicles for the transduction of antiviral gene constructs. Our initial evaluation of this approach suggests that cell stimulated in this manner are not susceptible to infection with laboratory or clinical isolates of HIV.

Table 12 Effect of Antiviral Gene Constructs on HIV-1 Reverse Transcriptase Production											
Cell Line (SRA #)	pJM #	Description	CD4	Total Cell Count	Cells/Well	% of control growth	RT/Sample	Sample Vol.	RT/Well	RT/10e5 Cells	% Control
Virus = 8119											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	2366667	100.00	473333	180892	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	647667	100.00	129533	52161	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	154618	23.87	30924	16205	31.07
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	56249	8.68	11250	3857	7.39
Virus = RF											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	3475000	100.00	695000	265605	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	536500	100.00	107300	43208	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	664833	123.92	132967	69677	161.26
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	308667	57.53	61733	21166	48.99
Virus = IIIb											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	2793333	100.00	558667	213503	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	493333	100.00	98667	39732	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	150840	30.58	30168	15809	39.79
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	31518	6.39	6304	2161	5.44
CL097 is control for CL098 and 99.											

Table 13 Summary of Antiviral Effects of Gene Constructs in A3.01 Cells

Cell Line (SRA #)	pJM #	CD4	Total Cell Count	Cells/Well	% of control growth	p24/ml	% Control	p24/Well	p24 /10e5 Cells	% Control
490.GT0042	BK89	+	1.24E+06	4.12E+05	94	10	0.00	2	0	0.00
490.GT0043	321neo	-	9.23E+05	3.08E+05	70	1028	0.12	206	67	0.17
490.GT0044	321neo	+	1.52E+06	5.08E+05	116	547167	64.80	109433	21542	55.83
490.GT0045	357B	+	1.16E+06	3.86E+05	88	40909	4.85	8182	2121	5.50
490.GT0047	324	+	8.97E+05	2.99E+05	68	547072	64.79	109414	36593	94.84
490.GT0048	322neo	+	8.28E+05	2.76E+05	63	711000	84.21	142200	51522	133.53
490.GT0049	320neoA	+	1.02E+06	3.40E+05	78	1150148	136.22	230030	67656	175.35
490.GT0050	311	+	1.42E+06	4.72E+05	108	791833	93.78	158367	33529	86.90
490.GT0051	292neoA	+	1.21E+06	4.03E+05	92	497333	58.90	99467	24682	63.97
490.GT0052	160-2	+	1.06E+06	3.53E+05	81	2050000	242.80	410000	116038	300.75
490.GT0053	136	+	1.18E+06	3.94E+05	90	913333	108.17	182667	46323	120.06
490.GT0054	290	+	1.26E+06	4.20E+05	96	646448	76.56	129290	30759	79.72
490.GT0056	188	+	1.11E+06	3.68E+05	84	1171500	138.75	234300	63611	164.87
490.GT0057	253	?	1.21E+06	4.03E+05	92	1111	0.13	222	55	0.14
CL085	322	-	9.49E+05	3.16E+05	72	522	0.06	104	33	0.09
CL086	tat	+	1.05E+06	3.51E+05	80	71	0.01	14	4	0.01
CL087	362A	+	7.67E+05	2.56E+05	58	24118	2.86	4824	1887	4.89
CL088	362B	+	6.24E+05	2.08E+05	48	422	0.05	84	41	0.11
CL089	362A	+	5.72E+05	1.91E+05	44	3188	0.38	638	334	0.87
CL090	354	+	1.04E+06	3.47E+05	79	2426	0.29	485	140	0.36
CL091	tat	+	9.10E+05	3.03E+05	69	384500	45.54	76900	25352	65.71
CL093	A3.01	+	1.31E+06	4.38E+05	100	844333	100.00	168867	38583	100.00

Table 14 Evaluation of Antiviral Gene Constructs

Tech = Louis Davis
Virus = HIV-RF

Cell Lines =	**GT77**	**GT78**	**GT79**	**GT80**	**GT81**	**GT82**	**GT83**	**GT84**	**GT85**	**GT86**
				TCID50/TREATMENT						
REPLICATES	100	100	100	100	100	100	100	100	100	100
1	71486	952000	1240000	37717	48099	313000	312000	12	670000	294000
2	60436	821000	187000	78042	29438	1310000	52397	14	855000	122000
3	72825	1460000	711000	54706	23278	91182	45591	13	955000	486000
4	62932	888000	149000	50377	138000	152000	34528	13	749000	195000
5	21542	1370000	544000	27061	116000	209000	88667	13	876000	119000
No cell control	3015	32005	37819	1227	2374	22536	12472	9	119000	21734
MEAN	57844	1098200	566200	49581	70963	415036	106637	13	821000	243200
+/-SD	20981.99	294606.86	445341.11	19266.76	52543.41	506925.07	116581.71	0.71	111939.72	153273.29
CV	0.36	0.27	0.79	0.39	0.74	1.22	1.09	0.05	0.14	0.63
% CONTROL*	117	2215	1142	100	9	51	13	0	100	30

Virus = HIV - 8119

Cell Lines =	**GT77**	**GT78**	**GT79**	**GT80**	**GT81**	**GT82**	**GT83**	**GT84**	**GT85**	**GT86**
				TCID50/TREATMENT						
REPLICATES	100	100	100	100	100	100	100	100	100	100
1	129000	587000	1190000	35618	8552	100000	59421	24	55545	310000
2	41688	546000	402000	20254	14598	59654	>>>>>>>	22	64976	158000
3	20726	761000	315000	45082	22161	276000	172000	20	71446	311000
4	203000	579000	475000	40372	32288	331000	>>>>>>>	24	78011	650000
5	83574	715000	225000	70514	18334	60486	20169	29	67253	544000
No cell control	11468	17327	41062	1573	411	9264	2294	21	1919	9188
MEAN	95598	637600	521400	42368	19187	165428	83863	24	67446	394600
+/-SD	72985.55	94344.05	385321.03	18290.29	8879.34	128571.03	78811.38	3.35	8297.46	198541.68
CV	0.76	0.15	0.74	0.43	0.46	0.78	0.94	0.14	0.12	0.50
% CONTROL*	226	1505	1231	100	28	245	124	0	100	585

Table 15 Evaluation of Antiviral Gene Constructs

Tech = L. Davis
Virus = HIV-1RF

Cell Lines =	GT0096	GT0095	GT0094	GT0093	GT0092	GT0091	GT0090	GT0089	GT0088	GT0087
				TCID50/TREATMENT						
REPLICATES	100	100	100	100	100	100	100	100	100	100
1	7951	202000	93966	392000	133000	465000	794000	185000	1168	331
2	8332	195000	74147	568000	217000	295000	306000	>>>>>>>	>>>>>>>>	732
3	>>>>>>>	270000	19434	53310	138000	99319	190000	257000	871	>>>>>>>
4	>>>>>>>	216000	64277	>>>>>>>	>>>>>>>	432000	531000	318000	1231	427
5	>>>>>>>	404000	101000	584000	214000	165000	1160000	81600	827	387
No cell control										
MEAN	8142	257400	70565	399328	175500	291264	596200	210400	1024	469
+/-SD	269.41	87073.53	32173.92	246533.40	46249.32	160304.60	390659.95	101626.64	204.78	179.54
CV	0.03	0.34	0.46	0.62	0.26	0.55	0.66	0.48	0.20	0.38
% CONTROL*										

Virus = 8119

Cell Lines =	GT0096	GT0095	GT0094	GT0093	GT0092	GT0091	GT0090	GT0089	GT0088	GT0087
				TCID50/TREATMENT						
REPLICATES	100	100	100	100	100	100	100	100	100	100
1	222000	62025	565000	169000	474000	734000	878000	1160000	91	991
2	57187	156000	201000	224000	501000	477000	839000	651000	538	1972
3	15103	103000	11392	>>>>>>>	907000	261000	725000	441000	781	>>>>>>>
4	50129	19831	207000	>>>>>>>	708000	577000	1000000	587000	380	>>>>>>>
5	186000	113000	40278	239000	646000	1050000	764000	837000	486	>>>>>>>
No cell control										
MEAN	106084	90771	204934	210667	647200	619800	841200	735200	455	1482
+/-SD	91682.21	51858.74	220361.85	36855.57	175047.71	295412.76	107283.27	276778.25	251.12	693.67
CV	0.86	0.57	1.08	0.17	0.27	0.48	0.13	0.38	0.55	0.47
% CONTROL*										

3. Antiviral Drug Working Group

The primary responsibility of this group is to utilize an *in vitro*, peripheral blood mononuclear assay to determine the incidence and clinical significance of AZT resistance in patients with HIV disease being treated with AZT (RV43 study). During the period 10/92-10/93, this group also participated in the following additional studies:

1. RV65 - to determine the time course of development of resistance to an experimental compound (here called compound A) in patients with HIV isolates demonstrating *in vitro* resistance to AZT.

2. RV79 - An ACTG/NIAID sponsored clinical evaluation of the codon 215 genotypic assay. To date more than a hundred specimens have been evaluated for the presence or absence of this phenotype.

3. CPCRA (007/014) - prospective evaluation of the development of *in vitro* anti-retroviral resistance in HIV-1 isolates obtained from patients participating in the CPCRA Combination Nucleoside clinical trial.

4. The Johns Hopkins University/MACS studies.

5. The Johns Hopkins University seroconverter study.

6. The *in vitro* testing of experimental anti-retroviral compounds using HIV-1 isolates.

The drug sensitivity assays performed on RV43 and RV65 isolates resulted in the determination of the *in vitro* drug inhibitory concentration of four anti-viral agents for each virus isolate tested. An example of a final report for an RV43 patient is presented in Table 16. During this period 268 drug sensitivity assays were performed and reported for RV43 patient isolates. Assays performed on the six patients enrolled in the RV65 study examined the *in vitro* resistance to AZT, ddC, ddI, and compound A. Thirteen assays were performed before this study was terminated.

For the additional studies, 187 CPCRA specimens were received and processed for virus isolation. It is anticipated that during the next fiscal year virus titration and drug sensitivity assays will be performed on these isolates. For the Johns Hopkins University MACS study the drug testing group received 52 vials of frozen cells from individual patients for virus isolation. Virus was isolated from 30 of these specimens and we were requested to determine the virus titration and *in vitro* resistance to AZT for 16 of these isolates.

For the Johns Hopkins University seroconverter study, we received 16 isolates for

Table-16: VIRUS ISOLATE NUMBER 102743

Date Received: 3/4/94

Date Virus Titration Set-up: 5/8/94

Titration Data

Assay Date	O.D. Cutoff	Number of + wells per Virus Dilution 16	64	256	1024	4096	16384	65536	$TCID_{50}$	4-Drug Plate Virus Stock Required
5/19/94	0.504	6	6	6	6	2	2	1	6472	0.530

Date Drug Sensitivity Set-up: 6/1/94

Date Drug Sensitivity Assayed: 6/8/94

Drug Sensitivity Data

AZT (uM)	$p24x10^5$	Fraction Affected
0	3.69	
0.001	3.35	
0.01	3.44	
0.1	3.52	0.05
1.0	2.01	0.46
5.0	0.25	0.93

IC_{50} = 0.8979

ddC (uM)	$p24x10^5$	Fraction Affected
0	3.69	
0.01	2.83	0.23
0.1	0.48	0.87
1.0	0.04	0.99

IC_{50} = 0.0247

ddI (uM)	$p24x10^5$	Fraction Affected
0	3.69	
0.1	3.33	0.10
1.0	2.86	0.22
5.0	2.46	0.33
10.0	1.40	0.62
25.0	0.07	0.98

IC_{50} = 2.5091

Compound A	$p24x10^5$	Fraction Affected
0	3.69	
0.03	3.63	0.02
0.3	0.09	0.98
1.0	0	
3.0	0	

IC_{50} = 0.0949

testing *in vitro* AZT resistance. Because of low virus titration results obtained for two of these isolates, assays could only be performed on 14 of these specimens.

Several experimental compounds were tested to determine their ability to inhibit replication of HIV isolates. These drugs were obtained from the laboratories of the Department of Applied Biochemistry of the Walter Reed Army Institute of Research, the Laboratory of Medicinal Chemistry at the National Cancer Institute, and private pharmaceutical companies. These compounds were tested by using isolates from RV43 patients and AZT-resistant and sensitive control virus isolates (Tables 19 and 20).

Drug Testing (10/1/93 - 9/30/94)

During this period, specimens were received and processed for RV43, CPCRA and RV79 as described above. The processing of these samples represented the establishment of 403 cultures for culture isolation and expansion. A summary of samples processed is presented in Table 17 below.

Table 17

Samples Received/Processed

Month	RV43	CPCRA	RV79
October	18	21	NA
November	14	28	NA
December	13	22	NA
January	18	15	NA
February	11	18	5
March	16	17	28
April	12	19	27
May	6	25	28
June	10	29	37
July	7	13	20
August	1	26	32
September	6	11	24
Total	132	244	201

Virus isolated from the RV43 specimens were further characterized by titration to determine the $TCID_{50}$ and subsequently tested for drug sensitivity as shown in Table 18. These assays represented the establishment of 24,888 cultures.

Table 18

Drug Plates/Titration Plates Set Up

Month	Titrations	Drug Plates
October	22	25
November	20	28
December	16	15
January	18	31
February	28	24
March	16	26
April	32	23
May	14	25
June	10	17
July	26	16
August	20	10
September	15	22
Total	237	262

Several experimental compounds were tested to determine their ability to inhibit replication of HIV isolates. These drugs were obtained from the laboratories of the Department of Applied Biochemistry of the Walter Reed Army Institute of Research (Dr. Peter Chiang) and private pharmaceutical companies. Fourteen drugs were tested Dr. Chiang using a total of 97 isolates (Table 19).

Table 19

Testing of Drugs from Peter Chiang

Number of Isolates Tested	Drug Tested
7	A
6	B
6	C
6	D
6	E
6	F
6	G
6	H
6	I
6	J
10	K
10	L
10	M
6	N

The results for testing these compounds are in Table 20.

Table 20

Isolate	Drug/IC_{50} (uM) AZT	A	Isolate	Drug/IC_{50} (uM) AZT	A
A012 Sensitive	0.0193	0.0126	**1**	1.8569	0.0106
A012 Resistant	2.0443	0.0043	**2**	1.9833	0.0534
A018 Sensitive	0.0211	0.0233	**014A**	0.0254	3.3393
A018 Resistant	1.6845	0.0012	**014B**	1.1124	3.8803
A012 Sensitive	0.0642	5.0223	**A018 Sen.**	0.0422	2.4652
A012 Resistant	2.3455	4.5246	**A018 Res.**	1.6435	3.9936

Drug/IC_{50} (uM)

Isolate	AZT	C	D	E	F
A012 Sen.	0.0647	1.4960	1.3453	0.6254	2.9348
A012 Res.	1.9895	3.3474	1.2253	1.2123	3.2264
A018 Sen.	0.0233	1.2334	2.7653	1.0394	2.3382
A018 Res.	1.6565	2.7685	1.8335	1.2235	2.2234
014A	0.0332	1.5467	0.9330	1.3393	2.8225
014B	1.1344	1.3336	1.3358	1.5325	2.1223

Drug/IC50 (uM)

Isolate	AZT	G	H	I	J
A012 Sen.	0.0431	1.5730	1.6794	1.8746	3.9333
A012 Res.	2.1745	1.1343	1.5643	1.7433	2.3344
A018 Sen.	0.0233	1.3244	1.8673	1.9333	3.5442
A018 Res.	1.8325	0.9745	1.1995	1.6766	3.2440
014A	0.0199	0.8846	1.3430	1.1023	2.4335
014B	1.0434	1.2348	1.5758	1.5235	3.2234

IC50 (uM)

Isolate	AZT	K	L	M
A012 Sen.	0.0236	0.0098	0.1951	0.1498
A012 Res.	2.0865	0.0020	0.1098	0.3426
A018 Sen.	0.0386	0.0117	0.1747	0.2013
A018 Res.	2.9652	0.0079	0.7004	0.0902
14A Pre	0.0239	0.0118	0.1218	0.0886
14A Post	1.7699	0.0011	0.0589	0.0966
1	1.4037	0.0196	0.9163	0.1439
2	0.0669	0.0076	0.1391	0.0598
3	2.1649	0.0388	0.2860	0.1604
4	0.5003	0.0039	0.0078	0.0076
5	0.0066	0.0047	0.0311	0.0091

	IC50 (uM)	
Isolate	AZT (uM)	N
A012 Sens	0.0416	0.0994
A012 Res	3.1675	0.1427
1	0.0237	0.2387
2	0.3868	0.1670
3	>5.0	0.0489
4	0.1362	0.0786

Three drugs were also tested using 7 virus isolates for Dr. Jean-Claude Schmitt of the Rega Institute for Medical Research in Leuven, Belgium.

A drug was provided by John S. Driscoll of the Laboratory of Medicinal Chemistry from the National Cancer Institute. Testing was performed with this drug and AZT, ddI, and ddC using 25 isolates. The results are in Table 21.

Table 21

	IC50 (uM)			
Isolate	AZT	ddI	ddC	FddA
A012 Sens	0.0102	1.6352	0.0237	3.2384
A012 Res	2.6932	2.9342	0.0264	5.2840
A018 Sens	0.0383	3.8212	0.0832	6.6324
A018 Res	1.8934	3.1274	0.0302	5.4948
1	0.0131	--	--	7.7237
2	0.0246	--	--	7.3186
3	0.1072	0.9760	0.0197	3.0404
4	4.1958	10.1743	0.0387	3.7096
5	0.7222	0.9145	0.0282	3.8120
6	0.0258	--	--	3.2504
7	>5.000	13.0262	0.1228	2.3546
8	0.3595	16.5500	0.0422	8.2713
9	0.0603	8.6825	0.0443	6.2096
10	0.2049	12.6664	0.1191	4.4720
11	0.0724	--	--	4.2535
12	0.0898	6.1405	0.3002	3.6967
13	0.5057	--	--	4.7201
14	>5.000	3.3547	0.0371	4.3677
15	0.0495	0.7313	0.1319	0.5207

16	>5.000	2.7274	0.0350	3.5251
17	0.0536	1.2533	0.0825	2.7990
18	0.0307	--	--	2.5998
19	0.4778	1.7625	0.2035	3.3426
20	>5.000	3.8062	0.0921	2.6549
21	0.0917	1.7053	0.1161	3.1623
22	>5.000	2.8649	0.1191	0.8675

Two drugs, provided by Dr. P. H. Riche, VIH-1 and were also tested against five isolates.

Johns Hopkins University provided 27 virus isolates for titration and AZT drug testing. SRA was able to determine $TCID_{50}$ from all 27 isolates but was only able to obtain AZT sensitivity data from 15 of these isolates. The data is in Table 22.

Table 22
Final Results of Hopkins Isolates

Specimen #	TCID50/200 ul	AZT IC50 (uM)
1	16384	0.0231
2	8192	No infection of cells
3	2048	0.0040
4	1618	0.0057
5	16384	0.0378
6	2592	0.0687
7	809	0.0093
8	4096	No infection of cells
9	512	0.0019
10	8192	0.0093
11	2048	0.0159
12	512	No infection of cells
13	512	0.0112
14	2048	No infection of cells
15	8192	No infection of cells
16	2048	No infection of cells
17	2048	No infection of cells
18	2592	No infection of cells
19	2048	0.0738
20	512	No infection of cells
21	8192	No infection of cells
22	10369	0.0456
23	512	No infection of cells
24	2592	0.0122
25	2048	0.0793
26	2048	No infection of cells
27	5185	0.9531

Development of a rapid drug screening assay

The current assays used to identify drug resistant HIV isolates require virus isolation, expansion and titration of the isolate followed by phenotyping. In order to reduce the time and cost, a rapid drug phenotype screening assays was developed. After a positive virus culture is confirmed, the procedure is as follows:

1. Resuspend the cells in the p24 positive culture (A1 Tube) and divide into three tube cultures each containing 0.800 ml of the resuspended cells. Label the tubes with SRA number and as AZT 0, AZT 0.2, or AZT 2.0

2. Into each of the three tubes, place 0.450 fresh co-cult media containing $2x10^6$ PHA-stimulated PBMCs.
3. Into the tube labelled AZT 0, place 1.25 ml fresh co-cult media. Into the tube labelled AZT 0.2, place 1.25 ml fresh co-cult media containing 0.4 uM AZT. Into the tube labelled AZT 2.0, place 1.25 ml fresh co-cult media containing 4.0 uM AZT. Final volume of all three tubes should be 2.5 ml.

4. Continue to maintain the cultures using standard procedures. On Day 4 replace the media in the culture tubes with either co-cult media, co-cult media with 0.2 uM AZT, or co-cult media with 2.0 uM AZT. On Day 7, refeed the cultures with $2x10^6$ PHA-stimulated PBMCs in the appropriate media, i.e. no AZT, 0.2 uM AZT, or 2.0 uM AZT. Save an aliquot of media from each tube on Day 4 and Day 7 refeeds for p24 assay.

The p24 (pg/ml) results of this assay using 22 isolates and comparison values to the conventional assay is shown in Table 23.

Table 23

	AZT Concentration (uM)			
Isolate	0	0.2	2.0	IC50 (uM) by ACTG/DoD
1	1024	0	0	0.1010
2	1136	0	0	0.0920
3	867	321	0	1.3947
4	1467	416	0	4.4568
5	1102	0	0	0.0206
6	1445	56	0	0.6692
7	1876	827	47	1.4245
8	1203	528	0	1.5471
9	2046	1002	116	2.3359
10	1422	0	0	0.1371
11	1127	0	0	0.0379
12	1876	18	0	0.1767
13	1443	0	0	0.0603
14	1154	139	0	0.5008

15	1889	477	0	0.2527
16	1322	264	18	0.5843
17	964	567	43	0.3595
18	1221	1316	867	>5.0000
19	1802	1765	675	4.4568
20	1556	556	110	1.6692
21	1677	627	97	2.6602
22	1765	760	119	1.3726

SUMMARY

A number of significant contributions to WRAIR's mission have been made by this contract during FY94. The most important of these is the extension of the 215 ARMS assay for genotypic resistance to a number of patients in a large-scale, nationwide clinical trial to assess the significance of this approach. Comparison of the genotypic approach with the classical phenotypic assays of drug resistance described in the section above will undoubtedly demonstrate the usefulness of the genotypic assay for clinical management of patient therapy.

SRA has developed an in vitro assay for the evaluation of antiviral gene constructs in established cell lines and is actively pursuing a system that will enhance the efficiency of transduction in PBMCs that will permit the study of primary isolates. Such a system will prove invaluable for the longterm culture and ex-vivo treatment of patient cells with antiviral genes.

Finally, the efforts of this contract, during the past year, have resulted in development of an improved system for the rapid and cost effective phenotypic analysis of resistance to antiviral drugs as described in the previous section.

During the coming fiscal year it is anticipated that SRA will take on additional responsiblities for the improvement of mutational assays as well as incorporating production level assays for viral burden.

REFERENCES

1. Kwok, S., D. E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky. 1990. Effects of primer-template mismatches on the polymerase chain reaction; Human immunodeficiency virus type 1 model studies. Nucleic Acids Res. 18(4):999-1005.

2. Newton, C. R., A. Graham, L. E. Heptinstall, S. J. Powell, C. Summers, N. Kalsheker, J. C. Smith, and A. F. Markham. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. 17:2503-2516.

3. Sommer, S. S., J. D. Cassady, J. L. Sobell, and C. D. K. Bottema. 1989. A novel method for detecting point mutations or polymorphisms and its application to population screening for carriers of Phenylketonuria. Mayo. Clin. Proc. 64:1361-1372.

4. Newton, C. R., C. Summers, L. E. Heptinstall, J. R. Lynch, R. S. Finniear, D. Ogilvie, J. C. Smith, and A. F. Markham. 1991. Genetic analysis in cystic fibrosis using the amplification refractory mutation system (ARMS): the J3.11 MspI polymorphism. J. Med. Genet. 28(4):248-251.

5. Miedzybrodzka, Z. H., K. F. Kelly, M. Davidson, S. Little, A. E. Shrimpton, J. C. Dean, and N. E. Haites. 1992. Prenatal diagnosis for the cystic fibrosis mutation 1717-1, G->A using arms. Prenat. Diagn. 12(10):845-849.

6. Wenham, P. R., C. R. Newton, and W. H. Price. 1991. Analysis of apolipoprotein E genotypes by the Amplification Refractory Mutation System. Clin. Chem. 37(2):241-244.

7. Lo, Y. M., W. Z. Mehal, B. P. Wordsworth, R. W. Chapman, K. A. Fleming, J. I. Bell, and J. S. Wainscoat. 1991. HLA typing by double ARMS [letter]. Lancet. 338(8758):65-66.

8. Larder, B. A., and S. D. Kemp. 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance go zidovudine (AZT). Science. 246:1155-1158.

9. Larder, B. A., P. Kellam, and S. D. Kemp. 1991. Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. AIDS. 5(2):137-144.

10. St Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, and B. A. Larder. 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science. 253(5027):1557-1559.

11. Larder, B. A. 1992. 3'-Azido-3'-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. Antimicrob. Agents. Chemother. 36(12):2664-2669.

12. Kellam, P., C. A. Boucher, and B. A. Larder. 1992. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. Proc. Natl. Acad. Sci. U. S. A. 89(5):1934-1938.

13. Boucher, C. A., E. O'Sullivan, J. W. Mulder, C. Ramautarsing, P. Kellam, G. Darby, J. M. Lange, J. Goudsmit, and B. A. Larder. 1992. Ordered appearance of zidovudine resistance mutations during treatment of 18 human immunodeficiency virus-positive subjects. J. Infect. Dis. 165(1):105-110.

14. Romero, D. L., M. Busso, C. K. Tan, F. Reusser, J. R. Palmer, S. M. Poppe, P. A. Aristoff, K. M. Downey, A. G. So, L. Resnick, et al. 1991. Nonnucleoside reverse transcriptase inhibitors that potently and specifically block human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. U. S. A. 88(19):8806-8810.

15. Baba, M., C. E. De, H. Tanaka, M. Ubasawa, H. Takashima, K. Sekiya, I. Nitta, K. Umezu, R. T. Walker, S. Mori, et al. 1991. Highly potent and selective inhibition of human immunodeficiency virus type 1 by a novel series of 6-substituted acyclouridine derivatives. Mol. Pharmacol. 39(6):805-810.

16. Cushman, M., and P. Sherman. 1992. Inhibition of HIV-1 integration protein by aurintricarboxylic acid monomers, monomer analogs, and polymer fractions. Biochem. Biophys. Res. Commun. 185(1):85-90.

17. Rich, D. H., J. V. Prasad, C. Q. Sun, J. Green, R. Mueller, K. Houseman, D. MacKenzie, and M. Malkovsky. 1992. New hydroxyethylamine HIV protease inhibitors that suppress viral replication. J. Med. Chem. 35(21):3803-3812.

18. Larder, B. A., and C. A. B. Boucher. 1993. Polymerase chain reaction detection of human immunodeficiency virus drug resistance mutations. In Press.

19. Tindall, K. R., and T. A. Kunkel. 1988. Fidelity of DNA systhesis by the Thermus aquaticus DNA polymerase. Biochemistry. 27:6008-6013.

20. Krawczak, M., J. Reiss, J. Schmidtke, and U. Rösler. 1989. Polymerase chain reaction: replication errors and reliability of gene diagnosis. Nucleic Acids Res. 17(6):2197-2201.

21. Eckert, K. A., and T. A. Kunkel. 1990. High fidelity DNA synthesis by Thermus aquaticus DNA polymerase. Nucleic Acids Res. 18(13):3739-3744.

22. Lundberg, K. S., D. D. Shoemaker, M. W. W. Adams, J. M. Short, J. A. Sorge, and E. J. Mathur. 1991. High-fidelity amplification using a thermostable DNA polymerase isolated from Pyrococcus furiosus. Gene. 108:1-6.

23. Quinnan, G.V. Immunology of viral infections. In: Textbook of Human Virology. ed. R.B. Belshe, 103-113, 1984.

24. Groopman, J.E., Benz, P.M., Ferriani, R., et al. Characterization of serum neutralization response to the human immunodeficiency virus (HIV). In: AIDS Res and Hum Retroviruses, 3(2): 71, 1987.

25. Wendler, I., Bienzle, U., and Hunsmann, G. Neutralizing antibodies and the course of HIV induced disease. In: AIDS Res and Hum Retroviruses, 3(2): 157, 1987.

26. Bottiger, B., Ljunggren, K., Karlsson, A., et al. Neutralizing antibodies in relation to antibody-dependent cellular toxicity-inducing antibodies against human immunodeficiency virus type 1. In: Clin Exp Immunol 73, 339, 1988.

27. Vujcic, L.K., Shepp, D.H., Klutch, M. et al. Use of sensitivie neutralization assay to measure the prevalance of antibodies to the human immunodeficiency virus. In: J. Infect Dis, 157: 1047, 1988.

28. Robert-Guroff, M., Goedert, J.J., Naugle, C.J., et al. Spectrum of HIV-1 neutralizing antibodies in a cohort of homosexual men: results of a 6 year prospective study. In: AIDS Res and Hum Retroviruses, 4(5): 343, 1988.

29. Arendrup, M., Ulrich, K., Nielsen, J.O., et al. HIV neutralizing antibodies: development and association with HIV-1 related diesease. In: Scand L Infect Dis, 21: 19, 1989.

30. Albert, J., Abrahaamsson, B., Nagy, K., et al. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. In: AIDS 4: 107, 1990.

31. Harada, S., Koyanagi, Y., Yamamoto, N. Infection of HTLV-III/LAV in HTLV-I carrying cells MT-2 and MT-4 and application in a plaque assay. Science 229:563-566, 1985.

32. Karpas A., W. Gillson, P. C. Bevan.. et al: Lytic infection by British AIDS virus and development of rapid cell test for antiviral antibodies. Lancet. 2:695-697, 1985.

33. Nara, P.L., Gerard, R.W., Matthew, G.A., et al. Absence of cytotoxic antibody to human immunodeficiency virus-infected cells in humans and its induction in animals after infection or immunization with purified envelope glycoprotein gpl20. Proc Natl Acad Sci USA 84, pp. 3797, 1987a.

34. Nara, P.L., Hatch, W.C., Dunlop, N.M., et al. Simple, rapid, quantitative, syncytium forming microassay for the detection of human immunodeficiency virus neutralizing antibody. AIDS Res Hum Retroviruses 3, pp. 283-302, 1987b.

35. Montefiore, D.C., Robinson, W.E., Schuffman, S.S., et al. Evaluation of antiviral drugs and neutralizing antibodies to human immunodeficiency virus by a rapid and sensitive microtiter infection assay. J Clin Microbiol 26:231-235, 1988.

36. Culliton, B. (1990): Gene therapy begins. Science 249, 1372.

37. W. French Anderson. (1992): Human Gene Therapy *Science* Vol. 256, 808-813.

38. Rosenberg, S.A., Aebersold, P. Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M.T., Yang, J.C., Topalian, S.L., Merino, M.J., Culver, K., Miller, A.D., Blaese, R.M. and Anderson, W.F. (1990): Gene transfer into humans - Immunotherapy of patients with advanced melanoma using tumor infiltrating lymphocytes modified by retroviral gene transduction. N. Engl. J. Med. 323, 570-578.

39. Buchschacher, G.L. (1993): Molecular targets of gene transfer therapy for HIV infection. JAMA, 269 (22), 2880-2886.

40. David Baltimore. (1988): Intracellular Immunization. *Nature* Vol. 335, 395-396.

41. Rhodes, A. and James, W. (1990): Inhibition of human immunodeficiency virus replication in cell culture by endogenously synthesized antisense RNA. J. Gen. Virol., 71, 1965-1974.

42. Joshi, S., Van Brunschot, W., Asad, S., Van Der Elst, I., Read, S. E. and Berstein, A. (1991): Inhibition of human immunodeficiency virus type 1 multiplication by antisense and sense RNA expression. J. Virol., 65, 5524-5530.

43. Chatterjee, S., Johnson, P. R. and Wong, K. K. Jr. (1992): Dual-target inhibition of HIV-1 in vitro by means of an adeno-associated virus antisense vector. Science, 258, 1485-1488.

44. Sczakiel, G., Oppenlander, M., Rittner, K. and Pawlita, M. (1992): Tat- and Rev-directed antisense RNA expression inhibits and abolishes replication of human immunodeficiency virus type 1: a temporal analysis. J. Virol., 66, 5576-5581.

45. Sarin, Prem S., Agrawal Sudhir, Civeira, Maria P, Goodchild, John, Ikeuchi, Tohru, and Zamecnik, Paul C. (1988): Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynocleoside methylphosphonates. *Proc. Natl. Acad Sci.* USA, 85, 7448-7451, 1988.

46. Sarver, N., Cantin, E. M. and Chang, P.S., Zaia, J.A., Lande, P.A., Stephens, D.A. and Rossi, J.J. (1990): Ribozymes as potential anti-HIV therapeutic agents. Science, 247, 1222-1225.

47. Rossi, J.J., Cantin, E. M., Sarver, N. and Chang, P.F. (1991): The potential use of catalytic RNAs in therapy of HIV and other diseases. Pharmac. Ther., 50, 245-254.

48. Stevenson, M., Meier, C., Mann, A. M., Chapman, N., and Wasiak, A. (1988): Envelope glycoprotein of HIV induces interference and cytolysis resistance in CD4+ cells: Mechanism for persistence in AIDS. Cell, 53, 483-496.

49. Buchschacher, G. L. Jr., Freed, E. O. and Panganiban, A. T. (1992): Cells induced to express a human immunodeficiency virus type 1 envelope gene mutant inhibit the spread of

wild-type virus. Hum. Gene Ther., 3, 391-397.

50. Steffy, K. R. and Wong-Staal, F. (1993): Transdominant inhibition of wild-type human immunodeficiency virus type 2 replication by an envelope deletion mutant. J. Virol., 67(4), 1854-1859.

51. Trono, Didler, Feinberg, Mark B., and Baltimore, David. (1989): HIV-1 gag mutants can Dominantly interfere with the replication of the wild-type virus. *Cell* , 59, 113 -120.

52. Haigh, Alison, Greaves, Richard and O'Hare, Peter. (1990): Interference with the assembly of a virus-host transcription complex by peptide competition. Nature, 344, 257-259.

53. Sullenger, B. A., Gallardo, H.F., Ungers, G.E. and Gilboa, E. (1990): Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. Cell, 63, 601-608.

54. Lisziewicz, J., Rappaport, J., and Dhar, R. (1991): Tat-regulated production of multimerized TAR RNA inhibits HIV-1 gene expression. New Biologist, 3, 82-89.

55. Lee, T.C., Sullinger, B. A., Gallardo, H. F., Ungers, G. E. and Gilboa, E., (1992): Overexpression of RRE-derived sequences inhibits HIV-1 replication in CEM cells. New Biologist, 4, 66-74.

56. Morgan, R. A., Looney, D. L., Muenchau, D. D., Wong-Staal, F., Gallo, R. C. and Anderson, W. F. (1990): Retroviral vectors expressing soluble CD4: A potential gene therapy for AIDS., AIDS Res. Hum. Retroviruses, 6(2), 183-191.

APPENDIX

I Summary of Assays and Services Provided in FY94

Area, Assay or Service Performed	Numbers Performed(to date indicated)
Drug testing and Virus Phenotyping (thru Aug. 31, 1994)	
- RV43, CPCRA and RV79 samples proccessed	536
- Number of co-cults established from above samples	383
- Number of expansions established from above co-cults	306
- Number of drugs tested for Dr. Chiang	14
- Number of isolates tested with Dr. Chiang's drugs	97
- Number of assays resulting from testing Dr. Chiang's drugs	1512

- Assays for development of rapid drug screen 110
- Virus titrations in support of drug studies 9324
- Total number of drug assays against 4 different drugs 13,680
- Total number of misc. drug assays for Dr. Mosca, Dr. Schmidt 1176

Molecular Biology

- Mutational Analyses (215) (thru Aug. 31, 1994)

  - RV43 RNA and DNA 215 assays 1083
  - RV79 RNA 215 assays 188
  - Seroconverter assays (RNA and DNA 215) 143
  - Others (RV77, FACs, Dr. Robb, etc 164

  Total assays 1578

- DNA Sequencing (Thru the end of June 1994)

  - Sequencing for 215 confirmation 15
  - Sequencing of drug resistant clones 13

  The numbers above represent sequence evaluation of 28 specimens containing 750 bases per evaluation or 750 x 28 = **21,000** total nucleotides evaluated.

- R & D (Assay Development and Validation)

  - Experiments to optimize and validate the mutational assay (approx. nine studies) Total assays 215

  - Extractions in support of Dr.Vahey (thru Aug. 31, 1994) 435

Virus Neutralization and Immunotyping

- Virus Titrations 7741
- Neutralization assays (AVEG and WHO) 5640
- R & D assays performed 8112
- SI/NSI assays performed 760
- Virus Expansions (thru August 31) 105

Antiviral Gene Therapy Evaluation

- Cell Cultures Maint./Mycoplasma Eval./Treat.

85

- Virus Expansions 5
- Virus Titrations 300
- Antiviral Gene Evaluations 8220
- Reverse transcriptase assays 7032

Support Services

- HIV Antigen Capture (p24 assay wells thru 8/31/94) 73,961
  See p24 well totals chart on next page (80)

This alone accounts for $169,053.71 of contract funds expended in the areas of drug phenotyping, neutralization assay development, immunotyping, and antivral gene therapy.

- Repository (number of vials added to contract inventory since start of fiscal year) 12,103

P24 Assay Well Totals

10/01/93 - 09/30/94

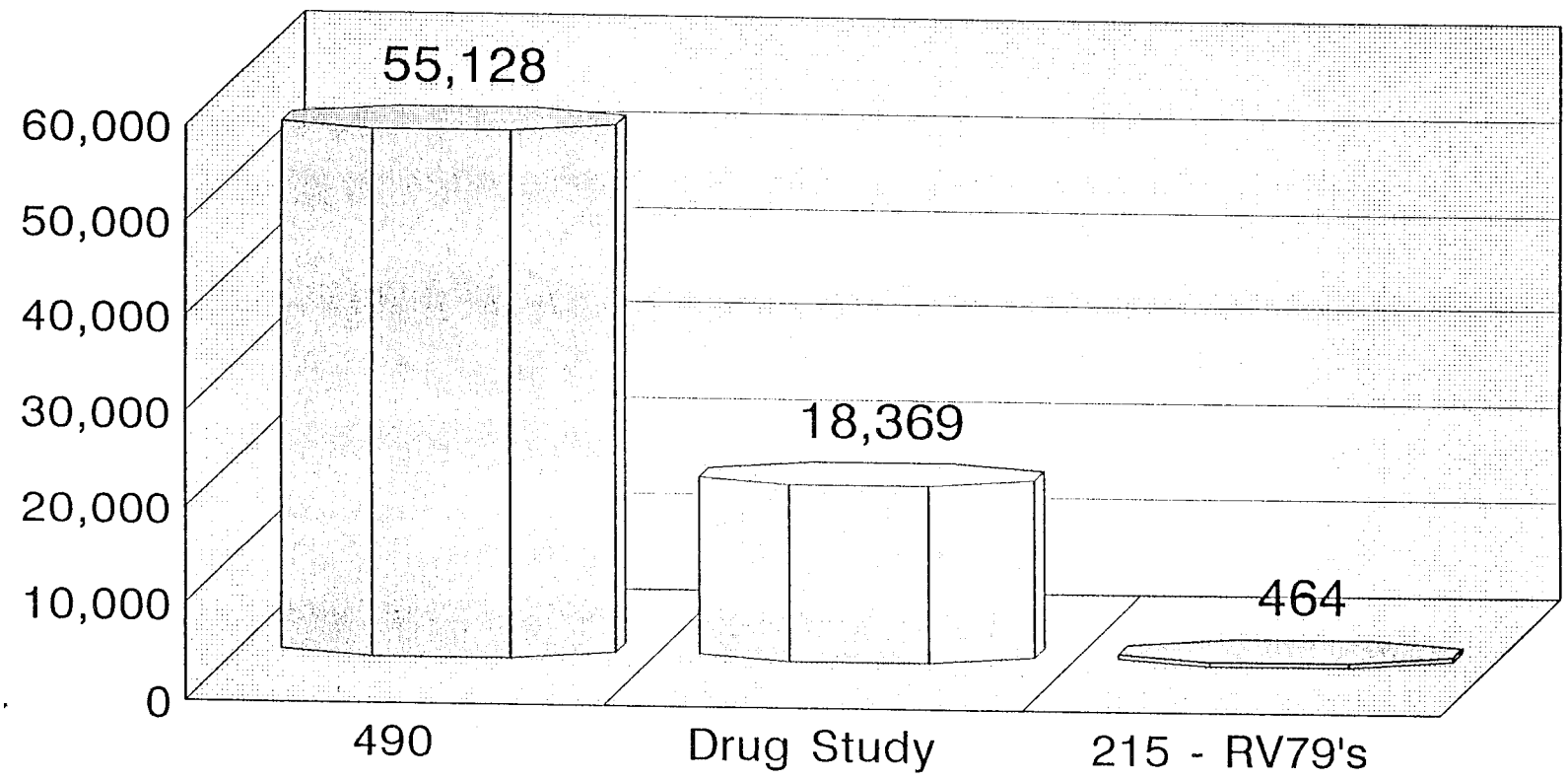


Plate Total	656.3	218.7	5.5

Contract

II Publications and Abstracts for FY94

Papers

1. Mayers DL, Mikovits JA, Joshi B, Hewlett IK, Estrada JS, Wolfe AD, Garcia GE, Doctor BP, Burke DS, Gordon RK, Lane JR, and Chiang PK. Novel anti-HIV-1 activities of 3-deaza-adenosine analogs: increased potency against azt-resistant HIV-1 strains. Proc. Nat. Acad. Sci.: Accepted for publication.

2. Mayers DL, Japour AJ, Arduino J, Hammer SM, Reichman R, Wagner KF, Chung R, Lane J, Crumpacker CS, McLeod GX, Beckett LA, Roberts CR, Winslow D, and Burke D. Dideoxynucleoside resistance emerges with prolonged zidovudine monotherapy. Antimicrob. Agents. Chemother. 38:307-314, 1994.

3. Winslow DL, Mayers D, Scarnati H, Lane J, Bincsik A, and Otto MJ. In vitro susceptibility of clinical isolates of HIV-1 to XM323, a non-peptidyl HIV protease inhibitor. AIDS 8:753-756, 1994.

Abstracts

1. Mayers DL, Lane J, and Weislow OS. Rapid screen of clinical specimens for drug resistant HIV phenotypes during virus isolation. Third International Workshop on HIV Drug Resistance, Kauai, Hawaii, August 1994.

2. Mayers DL, Yerly S, Perrin L, Imrie A, Cooper DA, Karney WW, Brown AE, Rakik A, Harris R, Gambel J, Weislow OS, Lennox JL, and Burke DS. Prevalence and clinical impact of seroconversion with AZT-resistant (AZTR) HIV-1 between 1988 and 1994. Second National Conference on Human Retroviruses and Related Infections, December 1994, Washington D.C.

3. Bernstein WB, St Louis DC, Profetto S, Weislow OS, Chung RCY, Wagner KF, Mayers DL and the RV43 Study Group. The timing and development of codon 215 mutations in plasma, CD4 and CD14 cells from HIV-infected patients on Zidovudine. Second National Conference on Human Retroviruses and Related Infections, December 1994, Washington D.C.

4. Mosca JD, Kaushal S, Larussa V, Kessler S, Gartner S, Kim J, Perera P, Yu Z, Ritchey D, Xu J, Rosenberg Y, St Louis D, Weislow O, Mayers D and Burke D. Human bone marrow-derived CD34+ cells as targets for gene therapy against HIV infection. Second National Conference on Human Retroviruses and Related Infections, December 1994, Washington D.C.

5. Mayers DL, Wagner KF, Chung RCY, Lane JR, Vahey MT, White FA, Ruiz NM, Hicks CBN, Weislow OS, Gardner LI, and Burke DS. Zidovudine (AZT) resistance is temproally associated with clinical failure in patients on AZT therapy. First National Conference on Human Retroviruses and Related Infections, December

1993, Washington D.C.

6. Mayers D, Erice A, Lane J, McCutchan F, Jabs D, Tudor-Williams G, Sannerud K, Weislow O, Page S, Felder K, and Balfour H. Foscarnet (FOS) resistant HIV isolates emerge during extended FOS therapy for CMV retinitis. 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, 1993.